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Proteomic analysis of skeletal muscle in dystrophic (mdx) mice subject to low-intensity endurance exercise

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DOTTORATO

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SUMMARY

The first aim of this study was to examine how low-intensity endurance exercise affects the regeneration process in rats with mechanical muscle damage. The rats were divided into three groups: sedentary, sedentary crush, crush trained. After the crush, to evaluate the regeneration process, rats were subjected to low-intensity endurance exercise by running on a motorized Rota-Rod for 5 v days/week for 4 weeks at progressively increasing loads.

At the end of the training period, the rats were sacrificed and the quadriceps muscle was collected for subsequent analysis by western blotting.

The reduction of regeneration process was evaluated by examining the protein expression of connexin-39 (Cx39), a specific gene expressed during regeneration process of injured muscles. While Cx39 was not expressed in sedentary rats, it was markedly increased in sedentary *crushed rats*, because of active degeneration/regenerating process, and dropped to very low levels in exercised *crushed rats*, suggesting a reduction of muscle regeneration process.

To evaluate the face of tissue regeneration compared to areas of necrosis, characteristics in the presence of damage muscle, histological sections were stained with hematoxylin-eosin. These sections have shown, with regard to the rats with crush trained a reduction of the face of tissue regeneration.

The second aim of the study was to conduct an analysis of the proteomic profile of mouse models of Duchenne muscular dystrophy, in order to understand what were the molecular mechanisms underlying the process of muscle regeneration increased from training. For proteomic analysis mdx mice were divided into three groups: wt mice, mdx mice sedentary and trained mdx mice.

The analysis of the proteomic profile evidenced that, among the phenotypes examined proteins that show a significant change in the levels of expression, are proteins that regulate the mechanism of oxidative stress, characteristic of the fibers dystrophic. In particular, we have identified three proteins that show variations between the phenotypes mdx sedentary and mdx trained mice: the three isoforms of carbonic anhydrase and superoxide dismutase-c.

The expression levels of the Sod-c decrease in the quadriceps muscle of mdx sedentary and increase levels of CAH-3; in the quadriceps muscle of mdx trained there is an increase in the Sod-c, and simultaneously a decrease of the levels of the CAH-3. Such changes may lead to a decrease of the formation of free radicals and then ultimately to a decrease in oxidative stress and therefore to a reduction of tissue damage.

This study has shown that specific low intensity endurance exercise induces a strong beneficial effect on the regeneration of damaged muscle and may have therapeutic value at least to decrease progression of muscular fibers necrosis.

INTRODUCTION

Skeletal muscle

Skeletal muscle is composed of multinucleated long cylindrical cells, which contract in response to stimuli volunteers to allow the movement of the entire body or parts of it. These cells originate from mesodermal precursors, myoblasts, which during embryonic development combine to give rise to multinucleated syncytia phones known as myotubes that subsequently differentiate into contractile muscle fibers. Despite the nuclei present within the muscle fiber, being exited from the cell cycle in an irreversible manner, they are in a permanent postmitotic state, the adult skeletal muscle is a highly dynamic tissue capable of regeneration after damage induced by exercise, by 'immobilization or by mechanical trauma or chemical substances. In response to exogenous stimuli or biological factors, muscle is, in fact, able to adapt increasing its size and the amount of contractile proteins. This implies an increase in the size of the fibers and their consequent production of force. The muscle remodeling occurs throughout life, although at different rates according to the stages of development, and is due to a particular type of stem cells located between the basal lamina and the sarcolemma of each fiber muscle called "satellite cells".

Discovered almost 50 years ago by Alex Mauro satellite cells were observed by him and described for the first time in the muscle fiber of frog. The cells were intimately associated with skeletal muscle fibers (MAURO, 1961) and positioned exactly between the membrane of the muscle fibers and the basal lamina.In adult muscle satellite cells are normally quiescent, but act as reserve cell population able to proliferate in response to any damage, thereby initiating the process of regeneration of skeletal muscle and giving rise to other satellite cells (Blaveri et al., 1999) . The study of satellite cells, until a short time ago identifiable only through accurate electron microscopy, is a result of very simplified following the discovery of the expression of particular protein factors from the aforementioned cells, but not by myonuclei in muscle fibers postmitotiche.Tra these factors: the M-cadherin cell adhesion molecule calcium - dependent, the C-met, the receptor for hepatocyte growth factor (HGF), nuclear factor miocitico (MNF) and Pax-7, transcription factor of type "paired box "(Hawke and Garry, 2001). Satellite cells appear on the edges of the muscle fibers in about seventeen days embryonic life, after they have formed the primary muscle fibers. Their origin is still not

known, but it seems that they derive from the dorsal aorta (Seale and Rudnicki, 2000). At the time of birth account for about 32%, but have decreased with age until it reaches a level of 1-5% in adults (Schultz and McCormick, 1994). The satellite cell activity is almost absent in adult skeletal muscle is not damaged and no myopathic (Decary et al., 1997), while satellite cells are activated as a result of muscle injury, when proliferate, differentiate into myoblasts fuse to form myotubes, which then differentiate into myofibers, or join the segments of the damaged muscle fibers to repair.

Connexins and gap junctions in skeletal muscle

The gap junctions (Figure) are specialized regions of membrane composed of aggregates of transmembrane channels that directly connect the cytoplasm of adjacent cells, and that allow the intercellular movement of ions, metabolites, and second messengers (Bruzzone et al., 1996b).

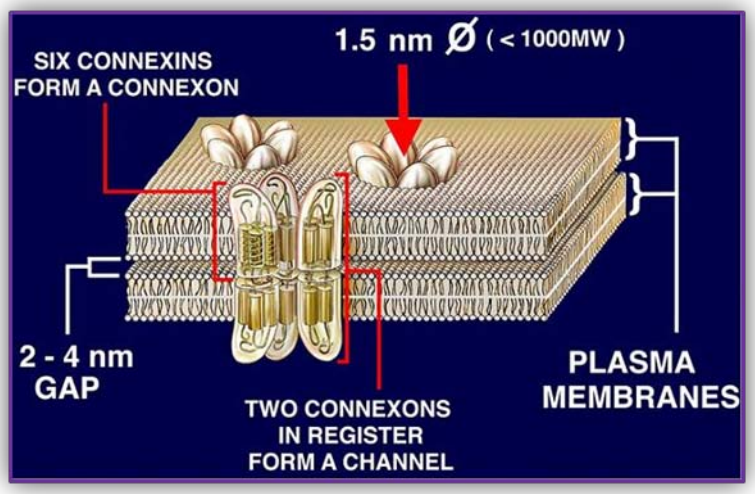


Fig.1: *Gap Junctions structure*

The passage of ions and small molecules through gap junctions, as second messengers (cAMP, IP3), produces an electrical coupling and metabolic cell. Each intercellular channel is formed by the junction of two hemichannels, or connexons, formed by assembling of hexameric protein subunits, called connexins and characterized by two distinct properties, the ionic conductance and permeability. Each connection can contain either a single type of connexins (homomeric), or different types of connexins (heteromeric); also intercellular each

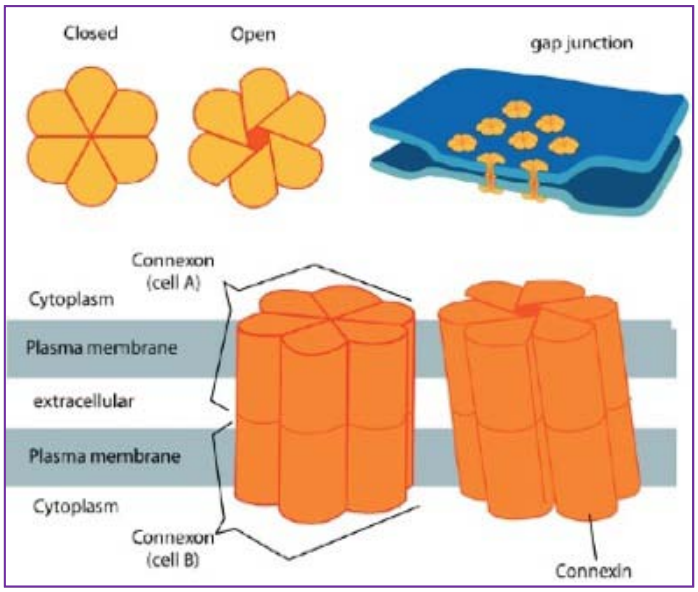


***Fig.2**: The figure shows the structure and functioning of connexons.*

channel can be defined homotypic or heterotypic (Bruzzone et al., 1996a), depending from the opposition of connexons that have the same or different molecular composition.

These intercellular channels are not always open, but is governed by several factors such as pH, the membrane potential or different pharmacological agents. Each connexin consists of 4 α-helical transmembrane domains, two extracellular loops, a cytoplasmic loop, an amino terminal cytoplasmic domain, and a carboxy terminal.

When two cells are placed close together, the connections from one cell can stick with their counterparts in the neighboring cell and thus form a gap junction channel. According to the cell type and the connexins expressed, the connexons can function as hemichannels, providing a pathway for the signaling tansmembrana, while the gap junction channels will be able to provide a pathway for direct communication between the cytoplasms of adjacent cells. This type of intercellular communication permits coordinated cellular activities, critical function for homeostasis of the organ during development and adult life of multicellular organisms**.**

The gap junctions participate in the regulation of various functions, including the contraction of the cardiac muscle and smooth muscle (De Mello, 1994; Miller et al., 1989; Page and Shibata, 1981; Spray and Burt, 1990), the transmission of neural signals at synapses electrotonic (Auerbach and Bennett, 1969; Bennett and Verselis, 1992; FURSHPAN and POTTER, 1959; Sotelo and Llinas, 1972), and cooperation in the development of metabolic and vascular organs. The permeability of intercellular channels to second messengers may also regulate the secretion by the exocrine pancreas is that endocrine (Bruzzone and Meda, 1988; Serre-Beinier et al., 2000) and plays a critical role in the formation during development (Caveney, 1985; Guthrie and Gilula, 1989).

The connexin gene family includes 20 members in mice and 21 members in the human genome. The connexins are commonly named according to their molecular weight (for example, Cx43 has a mobility of 43kDa), and their genes have been classified into 4-5 groups

(according to the sequence homology). Each has a different pattern of connexin expression and different regulatory properties. Nine of these connexins are expressed in the brain, but only the connexins Cx36 (Condorelli et al., 2000) and CX45 are expressed in neurons. These two connexins are widely expressed during embryogenesis and their expression persists even during the post-natal life, in a specific region of the central nervous system. After birth the Cx36 remains expressed in many areas of the brain and in particular in the olfactory bulb (Belluardo et al., 2000). The CX45, however, is highly expressed during embryogenesis and up to 2 weeks after birth in almost all regions of the brain. Following its expression is limited to the thalamus, the CA3 region of the hippocampus and cerebellum (Maxeiner et al., 2003). The other connexins present in the nervous system are exclusively expressed in glial cells (Condorelli et al., 2003).

The gap junctions are found in almost all tissues of mammals with the exception of adult skeletal muscle, while they are present in embryonic development (Balogh et al., 1993; Schmalbruch, 1982). This characteristic is due to an intrinsic property of skeletal muscle. The muscle fibers, in fact, must be electrically isolated from each other to avoid the spread of the current depolarization. Contrary to adult muscle tissue was observed that the myogenic progenitor cells are capable of adhering and merge in a highly coordinated and form gap junctions, suggesting a role of these channels in early muscle differentiation (Constantin and Cronier, 2000). The gap junctions, in fact, have been finding in skeletal muscle embryonic and neonatal chicken, mice and rats. The importance of intercellular communication mediated by gap junctions in myogenesis is further supported by a study of (Proulx et al., 1997) in which it was shown that after application of pharmacological blockers of gap junctions, myoblasts do not express myogenin or MRF4, both markers of terminal differentiation.

Electron microscopy studies have also demonstrated the presence of gap junctions between myoblasts and between myoblasts and myotubes during embryonic development. These connexins are: Cx39, Cx40, Cx43 and CX45 (Belluardo et al., 2005; von et al., 2004). In myoblasts, the expression of Cx43 is predominant and is a prerequisite for fusion (Squecco et al., 2006).It is expressed initially in the embryonic stage of 11 days in the Somita dermomiotomo. The Cx43 is expressed in many cell clusters and their expression varies according to the type of muscle. At the embryonic stage of 18 days, when the miotubo is fully formed, the expression of connexin 43 is restricted to cells spread along the newly formed myotubes. The CX45, instead, is widely expressed in many cells of the skeletal muscles in the

early stages of myogenesis, then its expression decreases after birth and is limited to cells along the myotubes, probably satellite cells.

The different times of the expression of the connexon is important for the proper differentiation of skeletal muscle, for example, the over-expression of Cx43 in cultured myotubes of mouse, under control of a viral constitutive promoter active, leads to a significant cell death during differentiation . The first signal of mRNA for Cx39 was instead observed to E12, at the level of myotomes. At the embryonic stage of 16 days, the Cx39 mRNA is expressed in all skeletal muscles during development, but around the 20th day, the expression of Cx39 in several muscles decreases and disappears within the first week of post-natal life (Belluardo et al., 2005), suggesting that this connexin is not involved in the process of post-natal growth of muscle mass. It was also shown that this connexin is expressed in adult skeletal muscle in regeneration phase after damage from trauma (Belluardo et al., 2005).

A recent study has also shown that in mice deficient for the Cx39, skeletal muscles is regularly and myogenesis, as the regeneration process, was not affected .

Muscle Regeneration

One of the most important processes that have been conserved during the evolution is the tissue regeneration, in wich interactions between infiltrating inflammatory cells and resident cells must be finely coordinated if homeostasis and functionally are to be restored. If something disrupted these interactions the result would be the failure of regeneration, and in the worst case this would lead to the death of the individual.(Soehnlein and Lindbom, 2010)

The skeletal muscle, is the most abundant tissue of the body, Skeletal muscles consist of muscle cells, networks of nerves and blood vessels, and connective tissues that connect

individual fibers into bundles, which form the muscle. The epimysium is the fibrous outer layer that surrounds the complete muscle, the perimysium surrounds the bundles of myofibers, and the endomysium (also called the basement membrane) surrounds individual myofibers (Huard et al., 2002). Myofibers are the basic structural elements of skeletal muscle and are composed of multiple fused myoblasts. Newly formed multinucleated fibers exhibit central nucleation, and once the nuclei move to a subsarcolemmal position they are called myofibers (Huard et al., 2002). The interior of a myofiber contains the sarcomeres, which are the basic functional units of skeletal muscle. The sarcomere consists of thick myosin filaments that interdigitate with thin actin filaments and is specialized to respond to neuromuscular signals. In response to these signals, the cell depolarizes and this leads to the release of calcium from the sarcoplasmatic reticulum.

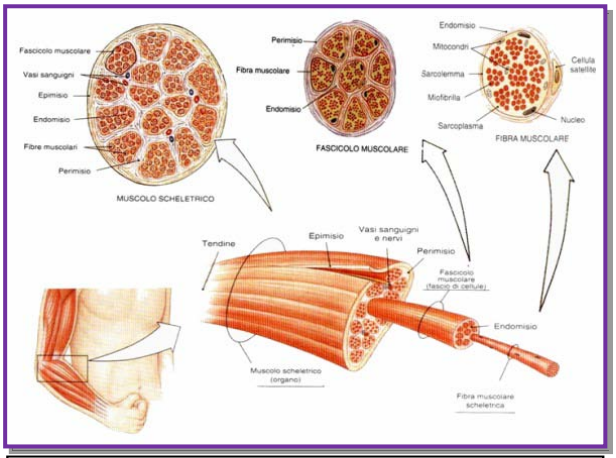


***Fig.3**.: Muscle structure*

It's important for a lots of physiological processes, so a loss of muscle functionality in acute or chronic conditions results in diminished mobility and strength, in addition to metabolic disorders, which can have potentiality lethal consequeces.

It may appen that a persistent myofibre degeneration and an inflammatory infiltration, as it occurs in DMD or when the extracellular matrix deposition is excessive, may bring to the substitution of the normal muscle architecture by fibrotic tissue.(Serrano and Munoz-Canoves, 2010)

Depending on the severity of the damage suffered by skeletal muscle depends on its own healing. However the healing process consists of three phases:

- The destruction phase
- The repair phase
- The remodeling phase. (Charge and Rudnicki, 2004; Huard et al., 2002).

The destruction phase is characterized by necrosis, hematoma formation, and the influx of inflammatory cells. In the repair phase, the necrotic debris is phagocytosed, and regeneration of myofibers occur through the action of SCs (*satellite stem cells)*. (Zammit et al., 2006b). At first, the SCs expressing Pax7 migrate to the site of injury, up-regulate the myogenic regulatory factors (MRFs) MyoD and Myf5, and become proliferative. From this moment on the SCs are also known as myoblasts. Subsequent differentiation of myoblasts is marked by the down-regulation of Pax7 and the up-regulation of the MRFs Mrf4 and Miogenin. (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994).Lastly, these differentiated myoblasts form multinucleated myofibers (*hyperplasia)* or fuse to damaged myofibers (*hypertrophy)* for muscle regeneration.(Charge and Rudnicki, 2004; Hawke and Garry, 2001).

Regulation of skeletal muscle regeneration

The skeletal muscle regeneration is a process regulated by a lots of factors, such as SCs, cytokines, growth factors, and neurotrophic factors that play a prominent role.

The satellite cell niche

The most common definition for a stem celle niche is "a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells with self renewing". (Ohlstein et al., 2004). In the SCs niche a lots of factors influenced the behavior of SCs, such as:

- Structural elements (basal membrane and myofibers);
- Products secreted by local cells, such as the interstitial cells, microvasculature, neuromuscular junction, immune cells. (Gopinath and Rando, 2008).

The SC niche is surrounded by the basal membrane (BM) and adjacent differentiated myofiber. Also the microvasculature seems to play a role: in humans and mice, respectively 68% and 82% of the SCs are located 5µm from neighboring capillaries or vascular endothelial cells (EC).(Christov et al., 2007). Furthemore there is a correlation between the number of capillaries per muscle fiber and the number of SCs (Christov et al., 2007). This strongly suggests a correlation between SC and ECs during myogenesis.

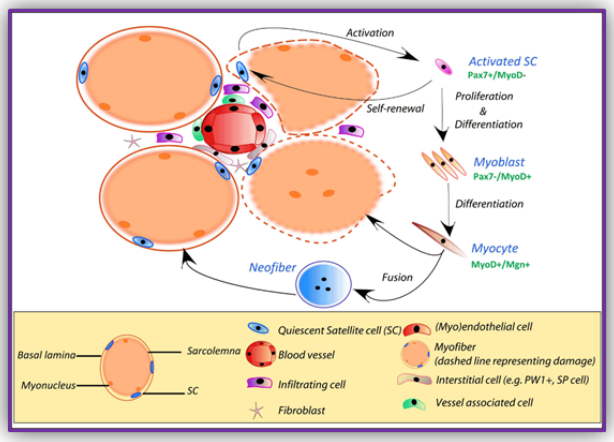


***Fig.4.** : The figure shows the activation of the*

Moreover, recent studies showed that macrophages, which are attracted upon injury, play a crucial role in skeletal muscle regeneration. In vivo macrophage suppression leads to incomplete skeletal muscle regeneration.(Segawa et al., 2008). Malerba (Malerba et al., 2009) and Segawa (Segawa et al., 2008), suggest that macrophage play an important role in that they act according two mechanism:

1. Macrophage can secret soluble factors affecting SCs;
2. Macrophage can interact with SCs by cell-cell contact, ant thereby protect them from apoptosis. (Chazaud et al., 2003)

Depending on their activity, macrophages play a dual role : pro-inflammatory macrophages that induce myogenic precursor cell proliferation; anti-inflammatory macrophages that induce differentiation and fusion of these cells.(Villalta et al., 2009).

The basis for the regulation of SC behavior is the attachment within their specific niche, wich is established trough cell-BM and cell-cell interactions. The basal side of the SCs expresses integrin α7β1, which links the cytoskeleton with laminin in the BM (Burkin et al., 2005) . The apical side expresses M-cadherin that attaches the SC to the adjacent myofiber (Cornelison and Wold, 1997; Kuang et al., 2008).Both attachment sites are essential for signal transduction betweenthe SC and the two flanking structures (Cornelison and Wold, 1997). Furthermore it has been suggested that M-cadherin plays a significant role in theattachment and fusion of myoblasts to form new and regenerate damaged myotubes. This is supported by a significant increase of M-cadherin in activatedSCs during skeletal muscle regeneration (Irintchev et al., 1994).

Growth Factor

Growth factor are crucial in SC regulation. Due to growth factor-activated intracellular signaling pathways (insulin growth factor-1, IGF-1) (Charge and Rudnicki, 2004; Philippou et al., 2007), both controlled up and down regulation of muscle specific genes occur.(Hawke and Garry, 2001). Immune cells and muscle cells after injury secreted a lots of growth factor, furthermore the vasculature, the SCs themselves and motor neurons are also responsible for growth factor production. There are also a group of growth factor that are defined “indirect” in that they are stored at the ECM (*extracellular matrix*) by binding proteoglycans e then they’re released from the ECM after skeletal muscle injury. To make this SCs have to increase the release of matrix metalloproteinases -2 and-9 after injury. These MMPs are involved in ECM degradation that liberates growth factors and cytokines. So, SCs can activate themselves indirectly .Furthermore, MMPs are involved in myoblast migration during

regeneration. (El et al., 2000; Kherif et al., 1999; Torrente et al., 2000). All these growth factors secreted together with that there're released from ECM, attract, activate, and induce differentiation the SCs. (Grefte et al., 2007; Huard et al., 2002). Between growth factors that promote the activation and subsequent differentiation of SCs we find:

- HGF;
- FGF-2 and -6;
- Vascular endothelial growth factor (VEGF);
- Platelet derived factor- I (SDF-I);
- IGF -1 and -2.

This factor together play a major role in myogenic proliferation and differentiation.
The major inhibitory factors in skeletal muscle regeneration are myostatin, transforming growth factor –α and β1 (TGF α and β1), and bone morphogenetic proteins (BMPs), wich are all members of the TGF – β superfamily.(Gordon and Blobe, 2008)

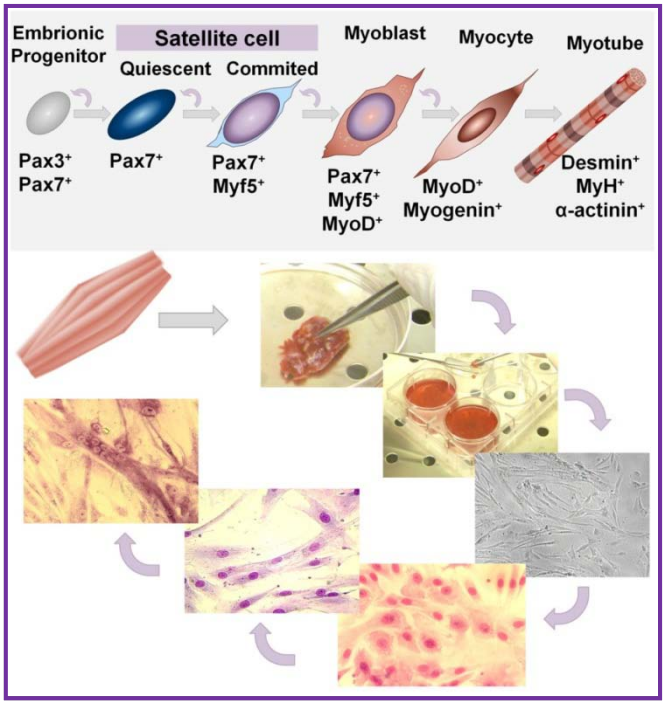


Fig.5: *The figure shows the differentiation of satellite cells into myotubes and the different factors expressed during the differentiation of SCs*

The family contains many regulatory factors, wich depending on the tissue, affect cellular behavior. Among these factors we find

myostatin. This factor is expressed in SCs and myoblasts. Myostatin release results in a down regulation of Pax3 and Myf5, and prevents the expression of MyoD. (Amthor et al., 2002). Myostatin may maintain SC quiescence and repress self-renewal through the induction of p21cip (McCroskery et al., 2003; Shi and Garry, 2006), wich is a universal inhibitor of cyclin-dependent protein kinase and thus a cell cycle inhibitor. (Jaumot et al., 1997)

Stem cells in skeletal muscle regeneration.

In addition to SCs many other stem cells show, at the level of skeletal muscle, myogenic capacity depending on the environment in which they are located.(Charge and Rudnicki, 2004). They can be divided into:

- Mesangyoblasts (vessel associated stem cells);
- Side population cells (SP cells);
- Muscle derived stem cells (MDSCs);
- Perycites;
- Cd 133+ stem cells. (Figure.)

Mesangyoblasts are vessel associated stem cells derived from the embryonic dorsal aorta and are able to differentiate into several mesodermal cell types including skeletal muscle. (Cossu and Bianco, 2003; Minasi et al., 2002).

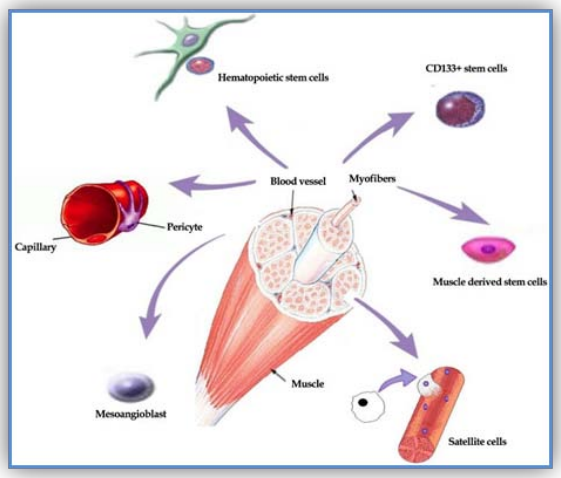


Fig.6: *The figure shows other cells that according to the microenviroment have myogenic capacity*

The heterogeneous muscle SP cells, are a rare, poorlydefined population in skeletal muscle, but they have the potential to give rise to both myocytes and SCs after IM injection (Asakura et al., 2002; Uezumi et al., 2006). Muscle SP cells are still present in Pax7_/_ mice, which exhibit a severe deficiency in SCs (Asakura et al., 2002).

MDSCs are a population of early myogenic progenitor cells which have, in contrast to SCs, multi-lineage potential.Pericytes are localized underneath the basal lamina of the microvasculature and interdigitate with the endothelial cells.

They give stability to the microvessels and also regulate blood flow and permeability of the vessels (Armulik et al., 2005). It has been suggested that pericytes are developmentally derived from mesoangioblasts (Cossu and Bianco, 2003; Dellavalle et al., 2007; Minasi et al., 2002).They become myogenic in vitro when differentiation is induced and contribute to muscle regeneration in dystrophic mice after intra-arterial injection (Boldrin and Morgan, 2007; Dellavalle et al., 2007). Unlike SCs, pericyte-derived myogenic cells express myogenic markers only in differentiated myotubes.

CD133+ cells circulate in the blood stream and they are able to differentiate, in vitro, into endothelial, hematopoietic, and muscle cell types (Torrente et al., 2004). CD133þ cells express adhesion molecules such as very late antigen-4 (VLA-4), which renders them capable to migrate through blood vessel walls.

The only relevant non muscle-derived stem cell, the hematopoietic stem cell (HSC), is also the most important multipotent stem cell participating in skeletal muscle regeneration after the SC (Charge and Rudnicki, 2004; Corbel et al., 2003; Ferrari et al., 1998). Among all these cells, the first cell type that participate to muscle regeneration is the satellite cells. Therefore these cells are a good candidate for the therapy of injured or desease muscle.(Zammit et al., 2006a).

Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD), is a genetic condition affecting predominantly boys that is characterized by fatal muscle weakness. While there is no cure, recent therapeutic advances have extended the lifespan of those with DMD considerably.

This pathology and the hologue condition in the mdx mice model, are caused by X-chromose gene mutations that lead to the absence of the protein dystrophin(Grounds et al., 2008; Hoffman and Dressman, 2001).Dystrophin is a subsarcolemmal component of a multi-molecular network (the dystrophin-glycoprotein complex, DGC) that ensures a physical linkage between the intracellular cytoskeleton and the extracellular matrix. The widely accepted theory is that dystrophin provides mechanical stability to myofibre during contraction. The absence of dystrophin triggers a complex and still unclear sequence of events that eventually leads to progressive myofibre degeneration, failing regeneration and fibrosis, thus resulting in muscle weakness and wasting.(Hoffman and Dressman, 2001).

This disease is caused by a mutation in the gene located in band Xp21 of the X chromosome that encodes a 427-kDa cytoskeletal protein called dystrophin, a protein essential for the maintenance of the integrity of cardiac and skeletal muscle cells. (Lovering et al., 2005).

The dystrophin gene spans approximately 2.5 Mb and is composed of 79 exons. In skeletal muscle, dystrophin is located on the cytoplasmic side of the sarcolemma and it is associated with glycoproteins and forms the dystrophin-glycoprotein complex (DGC). Structurally, dystrophin can be divided into four distinct domains: an amino-terminal domain, a large central rod domain, a cysteine-rich domain, and a carboxiyl-terminal domain. Other proteins of the DGC include dystroglycans (α e β), sarcoglycans (α,β,γ e δ), sarcospan, dystrobrevin (α), and syntrophins (α1, e β1).(Durbeej and Campbell, 2002)

The interactions of DGC proteins mediate the binding of the cytoskeleton to laminin-2 in the basal lamina of the extracellular matrix. The lack of the dystrophin protein interrupts the link between the extracellular matrix and the fiber cytoskeleton, causing sarcolemmal instability (muscle fibers lose their stability) and disorganization of DGC components during cycles of muscle contraction and relaxation. These events, in turn, lead to the exacerbated inflow of

calcium ions, activation of calcium-dependent endogenous proteases, inhibition of mitochondrial activity, and muscle fiber hyperconcontraction. A process of muscle regeneration then starts trough the activation of satellite cells, followed by the formation and maturing of myotubes in muscle fibers.

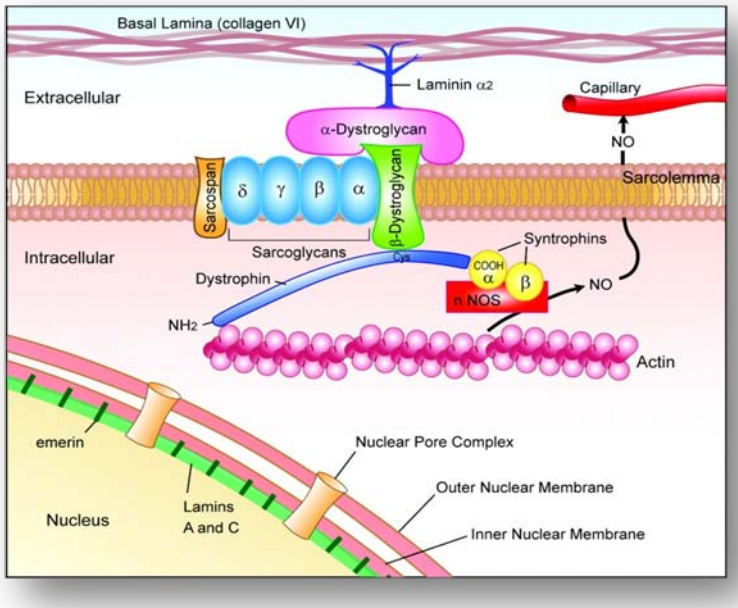


Fig.7: *Interactions of DGC proteins mediate the binding of the cytoskeleton to laminin-2 in the basal lamina of the extracellular matrix*

Various key events in the process have been identified, and many of them are related to muscle activity, such as recurrent states of ischemia due to impaired nitric-oxide induced vasodilatation, alterations in calcium homeostasis via mechano-sensitive pathways, an early and self- sustained inflammatory response paralleled by oxidative stress, and an impaired balance between protein synthesis and degradation.(Rando et al., 1998)

Fibrosis in DMD

Generally, fibrosis is referred to as replacement of normal tissue with scar tissue. This means that fibrous connective tissue is the result of reactive or reparative process.

As seen above, the genetic cause of DMD is an x-chromosomal mutation of the dystrophin gene. Dystrophin mechanically stabilizes myofibres by linking the cytoskeleton to the basal lamina through the dystroglycan complex; a dysfunction or a lack in the normal function of the dystrophin protein dues to an instability of muscle fibre membranes. As a result, the cells are less resistant to mechanical shear and prone to excess influx of electrolytes such as calcium and sodium.

This influx of electrolites produce an increase of intracellular sodium that leads to the ATP depletion, because the Na+/K+ pumps need to operate at full capacity. Water molecules

accompanying sodium produce cellular oedema. This gradient promotes calcium overload. Resulting in mithocondrial uncoupling and the production of reactive oxygen species (ROS) such as oxygen ions and peroxides. Downstream the result is an accumulation of inflammatory molecules such as cytokines.

Various stimuli including chemotaxis and mechanicals too, initiate the production of fibrous tissue. It is composed of extracellular fluid containing fibronectin, glycosaminoglycans and proteoglycans. These molecules can bind to water and therby influence the mechanic properties of the tissue.

Chronic tissue contracture is generated by a combination of cellular contraction and collagen fibre remodeling. Myofibroblasts actively contract via a calcium-dependent phosphorylation of the myosin light chain. Fibrocytes and myofibrobalsts usually are present for a time during tissue repair, such as in wound healing. In the case of DMD, activation of myofibrobalsts is persistent due to constant myofibre breakdown. This mechanism results in a altered production of ECM; the provisional ECM in fibrosis is different in composition from the ECM of normal tissue. In the early stage of fibrosis, the relative content of fibronectin and hyaluronan is higher than to non- injured tissue. This microenviroment creates a very hydrated matrix and facilitates cell migration.(Klingler et al., 2012)

Therapeutic approaches to muscular dystrophy

Muscular dystrophies are a group of heterogeneous diseases that primarily affect striated muscles throughout the body. Many of these myopathies are caused by mutations in genes that encode for structural proteins that link the cytoskeleton of muscle fi bers to the extracellular matrix. The absence of functional proteins results in destabilization of the muscle membrane, increased muscle fragility and degeneration, and progressive muscle wasting, all of which compromise patients' mobility and, in the severe disease forms (Emery, 2002) such as Duchenne muscular dystrophy (DMD1), lead to death. The course of the disease although it is progressive, varies from individual to individual. The diagnostic process can be initiated following a hit, sometimes random, increased creatine kinase (CK) in the blood, an enzyme that phosphorylates creatine and nonspecific marker of muscle damage. The diagnosis is based on two types of assessments: muscle biopsy and DNA analysis. Muscle biopsy can determine the histopathological often typical of a dystrophic process with degeneration, regeneration fibrale and fibro-fatty replacement and is the fabric on which to study the

presence / absence of dystrophin by immunohistochemical and biochemical analysis. Affected individuals can be diagnosed at birth on the basis of elevated serum levels of muscle enzymes. They exhibit muscle weakness by age 5, lose independent ambulation, and succumb to respiratory failure or cardiomyopathy in their late teens or twenties (Muntoni, 2003; Tyler, 2003).

In the absence of a cure for the disease, there are various therapeutic strategies which aim is to act on the secondary symptoms of the DMD for the purpose of limiting the progression and improve quality of life. These therapeutic interventions include:

- The use of steroids such as oxandrolone and prednisonehelps to increase protein synthesis and thus conserve muscle mass (Tidball and Wehling-Henricks, 2004; Zhao and Hoffman, 2004).;
- Proteolytic systems are targeted for decresing proteolysis in dystrophic muscle and slowing loss of muscle mass;
- Increased infl ux of calcium may be an option for increasing the activity of a calcium-dependent protease, calpain,in dystrophic muscle;
- Because overexpression of calpastatin, a natural inhibitor of calpain, reduces necrosis in *mdx* muscle (Spencer and Mellgren, 2002; Tidball and Wehling-Henricks, 2004), investigators have used β2-adrenergic agonists and pharmacophore to inhibit calpain activity (Burdi et al., 2006; Spencer and Mellgren, 2002). The application of immunosuppressants such as glucocorticoids and anti-TNFα antibody reduces the inflamm amatory responses associated with the disease and delays pathology (Grounds and Torrisi, 2004; Manzur et al., 2008)

However, despite the utility of these therapeutic treatments, side effects are encountered both on animal models and humans; therefore the use of these therapeutic interventions has been limited. The hope for the future, to correct the genetic defect present in DMD is that to introduce a functional gene for dystrophin in the muscles of patients with DMD. But the most important obstacle is represented by the huge size of the dystrophy gene. The normal gene replacement, therefore, is not presented as a possible approach and recently some studies have proposed alternative strategies, including the *exon skipping*. The skipping of specific exons aims to restore the reading frame of the dystrophin gene with the production of a protein internal missing but essentially functional, as is observed in the cases of Becker muscular dystrophy (Cossu, et al., 2007).

Has been also investigated pharmacological basis of a therapy that involves the up-regulation of the protein utrophin. This protein is an orthologue of dystrophin protein. Proof of principles studies in mdx mice (the animal model that is usually used to study DMD), have established that elevation of utrophin levels in dystrophic muscle fibers can restore sarcolemmal expression of the dystrophin associated complex (DAPC) members and alleviate the dystrophic pathology.

Another therapeutic strategy involves the use of stem cells. The stem cell therapy in an attractive method to treat musculardystrophy because only a small number of cells, together with a stimulatory signal for expansion, are required to obtain a therapeutic effect (Price et al., 2006). The clinical relevancecandidate stem cell population must be easily extracted, mustremain capable of efficient myogenic conversion, and when transplanted must integrate into the muscles allowing the functional correction of the dystrophic phenotype.(Farini et al., 2009)

These results, however, collide with a major obstacle for the development of a cell therapy in humans: the low efficiency of integration of the stem cells in damaged muscle. The skeletal muscle is in fact the most abundant tissue in humans, complicating the extensive spreading of the stem cells to the entire fabric. Furthermore muscle degeneration associated with muscular dystrophy is followed by a sort of scarring which reduces the blood supply to the muscle. A new study shows in fact that the induction of revascularization of muscle tissue can be a strategy to overcome this obstacle and be able to adopt the therapy MABs (mesangyoblasts therapy) with all patients (Gargioli et al., 2008).

Other therapeutic strategies include the development of specific programs of muscle exercise using as experimental animal models dystrophic mice.

The most widely used laboratory animal model for this type of experiments as it reflects the histopathology of Duchenne muscular dystrophy is the mdx mouse C57Bl/10ScSn (Bulfield et al., 1984). Mechanical function in mdx mice is not compromised and the muscle pathology is moderate and is more evident between the second and eighth week of life, a period characterized by the presence of necrotic foci, small areas of regeneration and a high level of plasma concentration of creatine kinase. Using different exercise regimens in an attempt to improve the function of the muscle showed that while an exercise excessive or improper use may cause further damage in the muscle doing worsen the condition (Brussee et al., 1997; Okano et al., 2005), an exercise in low intensity may make improvements to the muscle pathological (Hayes et al., 1993; Hayes and Williams, 1996; Hayes and Williams, 1998). The beneficial effects of regular exercise and low intensity have been reported after workout on

freewheel (Carter et al., 1995; Hayes and Williams, 1996), swimming and exercise of "endurance" to low intensity (Hayes and Williams, 1998).

Mdx mice as a model for the study of Duchenne muscular dystrophy

The mdx (X chromosome-linked muscular dystrophy) mouse is an experimental model widely used for the study of DMD, which is derived from a spontaneous mutation identified in theC57BL/10-ScSn mouse line. Like dystrophic humans, these animals carry a genetic mutation that causes the lack of dystrophin anan intense inflammatory process in muscles. However, mdx mice present efficient muscle regeneration and reduced fibrosis. For their characteristics mdx mice were used for the study of Duchenne muscular dystrophy and for the testing of biomedical techniques that reduce the damage of the fibers. In this context mdx mice, have been used to assay the effects of different exercise programmes on the function and morphology of dystrophic muscle.

Recovery of Damaged Skeletal Muscle in mdx Mice through Low-intensity Endurance Exercise.

The lack of dystrophin in *mdx* mice leads to cycles of muscle degeneration and regeneration processes. Various strategies have been proposed in order to reduce the muscle-wasting component of muscular dystrophy, including implementation of an exercise programme.

In a previous work conducted in our laboratory, (M. Frinchi, F. Macaluso, A. Licciardi , V. Perciavalle , M. Coco , N. Belluardo , G. Morici G. Mudò), it was demonstrated that an exercise of low-intensity endurance produces beneficial effects in dystrophic muscle of *mdx* mice. Moreover, in the same work, it was evaluated and quantified for the first time the regeneration process in the dystrophic muscle of sedentary and exercised *mdx* mice by monitoring the expression of connexin 39 (Cx39), a specific gene involved in skeletal muscle regeneration process.

The connexins (Cxs) are a multi gene family with at least 21 members in the human genome involved in gap junction channels formation in adjacent cell membranes by the docking of 2 hemichannels (connexons), each consisting of 6 Cxs subunits. And our research group has cloned the gene for connexin 39 by demonstrating that it is expressed only in muscular tissue and selectively expressed during embryonic skeletal muscle development, absent in the adult muscle and expressed again during the process of muscle regeneration.(Belluardo et al., 2005). From the study conducted in our laboratory, it was demonstrated that in muscle of *mdx* mice, endurance exercise produce beneficial effects, leading to remarkable recovery of

damaged muscle. These findings were supported by the results of the analysis and evaluation by histomorphological wb levels of cx39.

AIMS

First aim

Effects of a low-intensity endurance exercise on skeletal muscle of rats with muscle injury from mechanical trauma

In muscle biopsies from patients with Duchenne Muscular Dystrophy is possible to observe the areas of necrosis or degeneration of muscle fibers, often observed in clusters surrounded by macrophages and lymphocytes, and areas with small immature fibers with central nuclei which represent areas of regeneration, resulting in a balance between necrotic and regenerative processes (Brunelli et al., 2007; Grounds and Torrisi, 2004; Hodgetts et al., 2006; Radley et al., 2007; Spencer and Mellgren, 2002).

As described in the introduction, several strategies have been proposed in an attempt to reduce muscle degeneration characteristic of the disease, (Odom et al., 2007) (Carter et al., 1995; Hayes and Williams, 1996) (Hayes and Williams, 1998) and in the present study we wanted to study as an exercise in "endurance" low intensity, could facilitate the process of muscle regeneration in rats with injury mechanical trauma.. The work was devoted to the study of the process of muscle regeneration in rats that have suffered mechanical damage to the muscles after training.

Second Aim

Rating proteomic pattern of protein expression in wild type mice, mdx sedentary mdx trained mice to identify some important proteins involved in skeletal muscle regeneration.

Individual skeletal muscle protein are difficult to isolate and analyse biochemically due to the rigid structure of muscle fibers and the high density of contractile proteins and intracellular membrane systems.

Because of the high frequency of genetic muscle disordes there is considerable interest in studying protein expression and protein- protein interactions in normal versus diseased skeletal muscles. Often the identification of the primary genetic abnormality and the elucidation of secondary changes may not lead to a comprehensive understanding of the cellular downstream events that render muscle fibres more susceptible to necrosis. In conjunctions with findings from gene expression profiling studies, proteomics can be used to study how specific mutation can perturb the global protein expression pattern in muscle.(Doran et al., 2006)

Into the possible therapeutic approaches, a much debated about the role of exercise in the progression of Duchenne muscular dystrophy. Although in the literature are present conflicting data, the study conducted in our laboratory directed by Prof.re Belluardo, has shown that aerobic exercise of short duration and low intensity plays a role in slowing the progressive muscle degeneration that is characteristic of this pathology, and appears to facilitate the formation of new muscle fibers. In fact, subjecting the mdx mice to a workout of short duration and low intensity and comparing the histological sections of quadriceps obtained from mdx trainede mice, from mdx sedentary mice and from wild type mice, there was a substantial decrease of the areas of necrosis, in especially after 30 days of training and with a very high significance. So we wondered what were the molecular causes of these beneficial effects due to training in mdx mice, and such variations could exist in the pattern of protein expression in dystrophic muscle tissue subjected and not subjected to an exercise in endurance.

Materials and Methods

Animals

For the following study were used adult male Wistar rats (350 g bw) adult male mice and wild-type (C57BL/10ScSn from Harlan Italy). The animals were kept in a temperature (24 ± 2 ° C) and humidity (60%) and controlled with a period of alternating light-dark period of 12 h, with food and water ad libitum. All experiments were conducted following the guidelines of national (DL no. 116, GU, suppl. 40, February 18, 1992), the international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1 , December 12, 1987, National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, NIH Publication no. 80-23, 1985 and Guidelines for the Use of Animals in Biomedical Research, Thromb Haemost 58: 1078 - 1084, 1987) and in accordance with the approval of the ethics committee of national and regional health surveillance.

Animal model for the study of muscle regeneration following damage

To analyze the process of tissue regeneration was performed a lesion at the level of the quadriceps and gastrocnemius muscles of rats. The animals were anesthetized prior and after skin incision and gastrocnemius muscle was discovered and mechanically compressed in the central area with a hemostat for 3s. Immediately after the parts have been saturated resected. The animals were sacrificed by an overdose of anesthesia in groups of three at different days after injury. The dissected muscle was frozen in cold isopentane, and stored at -70 ° C

Training protocol

Both wistar rats with muscle injury were divided randomly to the group of sedentary (SD) or trained (EX). In a very similar approach to be deficient rats muscle injury that wild type mice were assigned to the group of sedentary (SD) or that of trained (or Wt-EX-EX).

Regarding rats also have undergone a pre-training of 9 days to speed and increasing time in order to accustom to the movement. On the ninth day of training was carried out at the level of the lesion and quadriceps and gastrocnemius muscle was taken up training for 7 consecutive days. 24 hours after the end of the last training session the rats were sacrificed with an overdose of anesthesia.

Western Blotting

Each experimental group of animals was sacrificed with an overdose of anesthetic. The gastrocnemius muscle was taken with the aid of the stereomicroscope , frozen in isopentane and stored at -70 ° C. From these muscles were taken cryosections of 60µm and used for the assay of the levels of connexin 39 by western blotting. The muscle samples were homogenized in a cold buffer containing 50 mM Tris- HCL pH 7.4 , 150 mM NaCl , 1 % triton , 0.1 % SDS , H2O and a cocktail of protease inhibitors (P8340 , Sigma -Aldrich Srl , Milan, Italy) . The homogenate was left on ice for 30 minutes and then centrifuged at 13,000 rpm for 30 minutes at 4 ° C. The supernatants were stored at -20 ° C and aliquots were used for protein determination using the Lowry method (LOWRY et al , 1951). The samples with 30 µg of protein a marker for the molecular weights (161-0375 , Bio -Rad Laboratories Srl , Segrate (MI) , Italy) , were run on 8 % polyacrylamide gel at 100 V and transferred electrophoretically nitrocellulose membrane (Hybond -C- extra , GE Healthcare, formerly Amersham, Europe GmbH Branch - Italian , Milan, Italy) . After 1 h of incubation in 5% nonfat milk , the membrane was incubated overnight at +4 ° C with the polyclonal rabbit anti-Cx39 at a dilution of 1:1000 (N-20 sc -546 , Santa Cruz Biotechnology , CA , USA). After washing the membrane was incubated for 1 h at room temperature with a specific secondary antibody conjugated with horseradish peroxidase at a dilution of 1:5000 (Sc 4 200 , Santa Cruz Biotechnology) and the band corresponding to Cx39 was displayed with reagents chemo luminescent (ECL, GE Healthcare, formerly Amersham, Europe GmbH Branch - Italian , Milan, Italy) . The ECL films were developed in Kodak D19 developer and fixed (Eastman - Kodak , Rochester, NY, USA) , and densitometric evaluation of the bands was carried out through the measurement of the optical density (OD) using the NIH ImageJ software and the results are were expressed as arbitrary units.

Histomorphological analysis

Longitudinal sections of 10µm of gastrocnemius and quadriceps were cut at -20 ° C and adherent on glass slides coated with 3-aminopropyl etossisilano. Sections of frozen muscle were directly fixed in 4% paraformaldehyde and stained with hematoxylin-eosin. Were finally dehydrated with ethanol, cleared in xylene and mounted with Entellan (Merck, Darmstadt, Germany) to be examined under the optical microscope.

Statistical Analysis

The results were reported as mean ± SE. The analysis of variance (ANOVA) and Fisher's PLDS correction for post hoc comparison was used to test the difference between the wild type animals sedentary, wild type trained, dystrophic dystrophic sedentary and trained, both for the mice that for rats at different time points. The trend over time for each group was assessed with the paired t-test (Statview 5.0.1, SAS Institute, Inc., Cary, NC). The data was considered significant for values of $p < 0.05$.

Proteomic Analysis

Protein extraction from biopsies.

The extraction was carried out at 4 ° C in an ice bath. The tissue fragments were mechanically homogenized with the Politron in 50 mM Tris / HCl pH 7.5 with antibiotics (0.003% and 0.005% Penicillin Streptomycin), put to stir over-night at 4 ° C, and centrifuged twice at 10000 rpm for 20 min. The supernatant, containing the tissue protein, has been put to dialyse against millipore water at 4 ° C for 48 hours in several changes. Subsequently, the lysate was freeze-dried, resuspended in ISOT (8 M Urea, 4% CHAPS, 65 mM DTE and 40 mM Tris) and subjected to quantitative assessment of protein content.

Two-dimensional electrophoresis 2D-IPG

The protein extract was analyzed using isoelectric focusing in immobilized pH gradient (A. Gorg et al., 1988; Byellqvist B. et al., 1993). The isoelectric focusing was performed on strips ready, produced by Pharmacia, dehydrated and stored at -20 ° C, with non-linear gradient, from 4 to 10 with an expansion in the region between 5 and 7. The strips, 3 mm wide, 180 mm long and 0.5 mm thick, were rehydrated individually for 1 hour in a solution containing 8 M Urea, 4% CHAPS (3 - (3-cholamidopropyl dimethylamino) 1 - propane-sulfonate) , 1% DTE, 0.5% Resolite (anfoline pH 3.5-10) and traces of bromophenol blue 0.5%. To this solution was added an aliquot of the extract containing 45 micrograms of tissue protein, for a total volume of 350 µl.

The run was performed at 20 ° C , with a voltage which increases linearly from 200 to 3500 V during the first 4.5 hours and then stabilized at 8000 V for 8 hours. At the end of the electrophoretic run the strips were equilibrated in a solution consisting of 6 M Urea, 30 % glycerol , 2 % SDS , 0.05 M Tris / HCl pH 6.8 , 2 % DTE , for 12 min. , And then in the same buffer solution , replacing the 2 % DTE with 2.5% of iodoacetamide . The second dimension was carried out on polyacrylamide gel with a gradient of 9-16 % , with dimensions 18 x 20 cm and a thickness of 1.5 mm . The strips were made balanced slip between the glass containing the gel of the second dimension, using a hot solution of 0.5 % agarose dissolved in buffer scroll in order to establish a continuity between the gel : the agarose , in fact , cooling solidifies , making the strip stay in the right position . The buffer scroll is made from 0.125 M Tris, 0.96 M glycine and 0.5 M SDS . The run was performed with a constant current of 20 mA per gel , until the arrival of the reference dye at the lower edge of the gel.

Silver staining

The silver staining is the most sensitive staining method (about 1 ng of protein per spot) among those not radioactive. It is based on the specific binding of silver ions on the proteins previously oxidized and the subsequent reduction of these ions to metallic silver using a citric acid solution containing formaldehyde.

The gel after the run are subjected to the following steps:

- Washing with milliQ H2O for 5 min.;
- fixing in a solution of Ethanol 40% - 10% glacial acetic acid for 60 min.;
- fixing in a solution of 5% Ethanol - Glacial acetic acid 5% over-night;
- washing the gel in milliQ H2O for 10 min.;
- Sensitization in a solution of Glutaraldehyde 2% - Sodium acetate 0.5 M prepared using milliQ H2O at 4 ° C;
- Three washes of 10 min. each with milliQ H2O at 4 ° C;
- two washes of 30 min. each in 0.05% NDS (2.7 naftalendisolfonico acid), prepared using milliQ H2O at 4 ° C;
- 4 washes of 15 min. each in milliQ H2O at 4 ° C;
- staining with an ammoniacal solution of silver nitrate (AgNO3) prepared with 0.8% AgNO3, 1% NaOH and 1% NH3 at 32%;
- 4 washes of 4 min. each with milliQ H2O at room temperature;

- development with a solution of citric acid, 0.005% and 1% formaldehyde at 37% for about 12 min. until they are visible and distinguishable spots;
- Stop development with a solution of acetic acid at 5%.

Each operation must be carried out under gentle agitation

Computer analysis of the gels

The gels stained with silver nitrate were imported into a scanner, and subsequently subjected to computer analysis using the computer system IMAGE MASTER 2D PLATINIUM that is constituted by a set of algorithms, which allow to make both qualitative and quantitative analysis of the gels.

The first step in the analysis of the gel is computed to eliminate the artifacts dependent scanning process (shadows and bubbles are considered by the software as protein spots). Once you correct the image, the computer system defines the protein spots and their position in the image. Next, using as internal standards some "anchors", ie proteins with known pI and PM (eg. Plasma proteins), it was possible to calibrate the gel in order to convert the spatial coordinates of each spot in the respective values of pI and PM.

For comparison (gel "matching") More images electrophoretic software uses special algorithms to match the shape and position of the spots present on different gels.

This automatic action can be difficult in case of changes in migration between gels, and for this reason it is preferable to pair manually some reference spot, which serve as the starting point for the "matching". For a correct pairing enough about 10/15 anchors distributed over the entire surface of the gel.

The quantitative analysis was carried out, always with the aid of the software, considering the volume percentage (% Vol) of each spot, calculated by comparing the volume of each single stain to the sum of the volumes of all peptides present in the map to limit the possible differences due to a different silver staining between gels. The volume of each spot is calculated from the integration of the OD on its area.

RESULTS

FIRST AIM

Effects of the exercise of "endurance" low intensity on regeneration in the gastrocnemius muscle of rat subjected to mechanical damage

The rats were divided into four experimental groups: sedentary control, control train them, crush sedentary and trained. As shown in Table II, the trained rats were subjected to a workout for nine days until it reaches the desired exercise regime; ninth day was carried out the mechanical crush of the gastrocnemius muscle as described in materials and methods. After crush the workout was continued for another 7 days and 24 h after the last training session the rats were sacrificed to assess the levels of Cx39 in the trained compared to control lesioned but not trained.

Table II

GIORNI	TEMPO	RPM	VELOCITÀ
1	15 min	20 g/min	4 m/min
2	15 min	20 g/min	4 m/min
3	15 min	20 g/min	4 m/min
4	15 min	20 g/min	4 m/min
5	30 min	24 g/min	4,8 m/min
6	30 min	24 g/min	4,8 m/min
7	30 min	24 g/min	4,8 m/min
8	30 min	24 g/min	4,8 m/min
9	45 min	24 g/min	4,8 m/min
10	45 min	24 g/min	4,8 m/min
11	45 min	24 g/min	4,8 m/min
12	45 min	24 g/min	4,8 m/min
13	45 min	24 g/min	4,8 m/min
14	45 min	24 g/min	4,8 m/min
15	45 min	24 g/min	4,8 m/min
16	45 min	24 g/min	4,8 m/min

Recovery of skeletal tissue damaged by mechanical trauma in rats

Histomorphological analysis

The histological analysis of sections of gastrocnemius muscle tissue was performed in order to evaluate the effects of an exercise of "endurance" low intensity on the progression of regeneration process in rats with mechanicl trauma. The sections of muscle tissue were stained using hematoxylin –esoin method and they reveals a visible reduction of the necrosis area and an upgrade of the regenerating part in the muscle section coming from the trained rat.

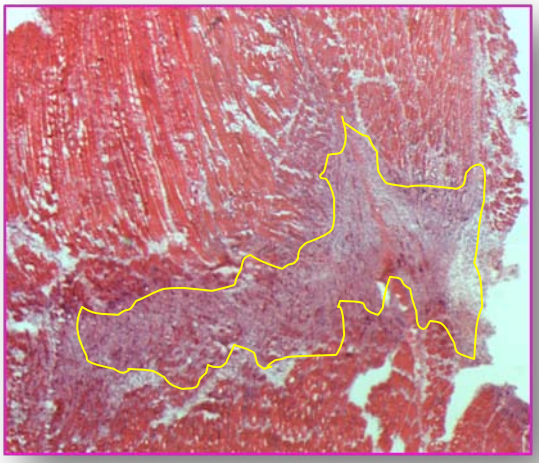


Fig. 9: Section of quadriceps muscle of sedentary rats.

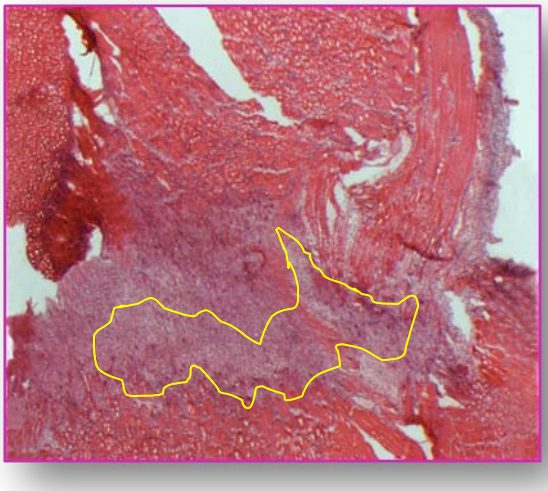
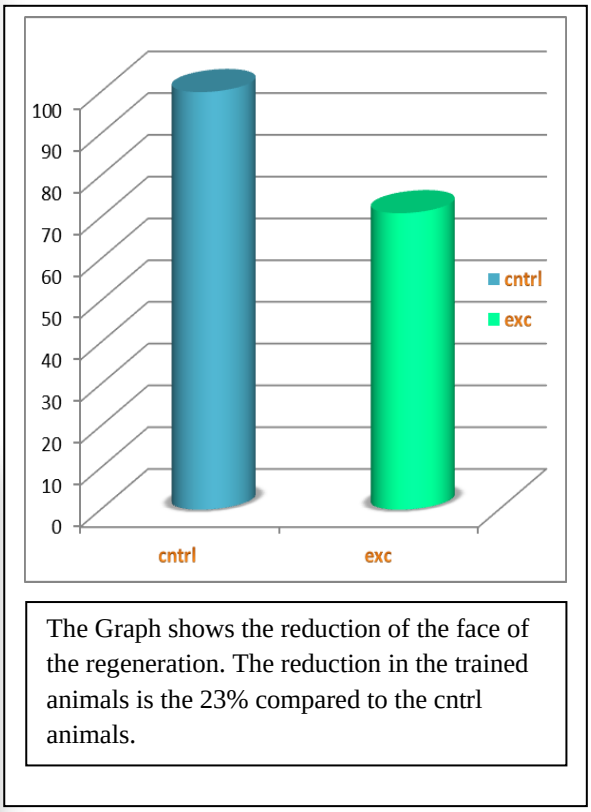


Fig. 10: Section of quadriceps muscle of trained rat



The Graph shows the reduction of the face of the regeneration. The reduction in the trained animals is the 23% compared to the cntrl animals.

Determination of the levels of Cx39 as a marker for assessing the degree of regeneration of the damaged muscle

In a previous work (Trovato-Salinaro et al., 2009) we demonstrated that Cx39 appears to be expressed exclusively in skeletal muscle tissue during embryonic development and one week after birth declines until it disappears, therefore, turns out to be not expressed in adult muscle. Such embryonic expression of Cx39 is involved in the aggregation and fusion of myogenic cells for the formation of myotubes and then miofibre muscle (Belluardo et al., 2005). As in adult skeletal muscle, different types of trauma promote the proliferation of satellite cells that differentiate into myoblasts to form new myofibers or repair the damaged myofibers, recapping the sequences of the embryonic processes, we hypothesized that the Cx39 could be re-expressed in the training phase of new myotubes regenerating.

In the figure below, the graph shows the results obtained by the western blotting analysis for the gastrocnemius muscle. Muscle control that does not have therefore suffered mechanical damage, Cx39 is absent. The presence of cx39 is also absent in the muscle of rats subjected to training. The expression of cx 39 is present and is elevated in the muscle of rats with mechanical muscle damage not trained. As we can see from the analysis of wb, in the muscle of rats trained with mechanical muscle damage, there is a reduction of cx39 expression. This reduction means that endurance training leads to an acceleration of the process of muscle regeneration and damage repair muscle. Altogether this model confirms that, during muscle regeneration following the crush, there is a transient activation of Cx39 in the damaged area and its expression testifies an active regenerat process (Belluardo et al., 2005).

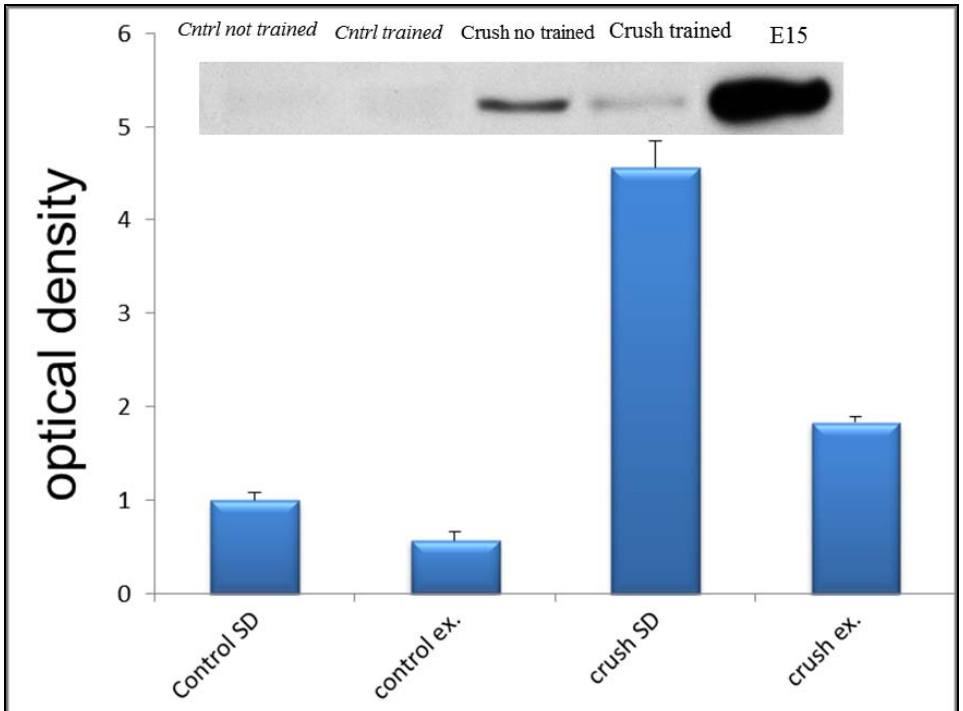


Fig.9: *Western Blotting analysis of the levels of the Cx 39 expression in rat's muscle with mechanical damage and subjected to training. The western blotting analysis shows that in rat not trained with crush there's an elevated expression of the Cx39 compared with rats with crush undergo training.*

SECOND AIM

Rating proteomic pattern of protein expression in wild type mice, mdx sedentary mdx trained mice to identify some important proteins involved in skeletal muscle regeneration.

Were identified 73 spots corresponding to 31 different proteins indicated in the underlying reference map by their respective code of access to the bank UNICOD.

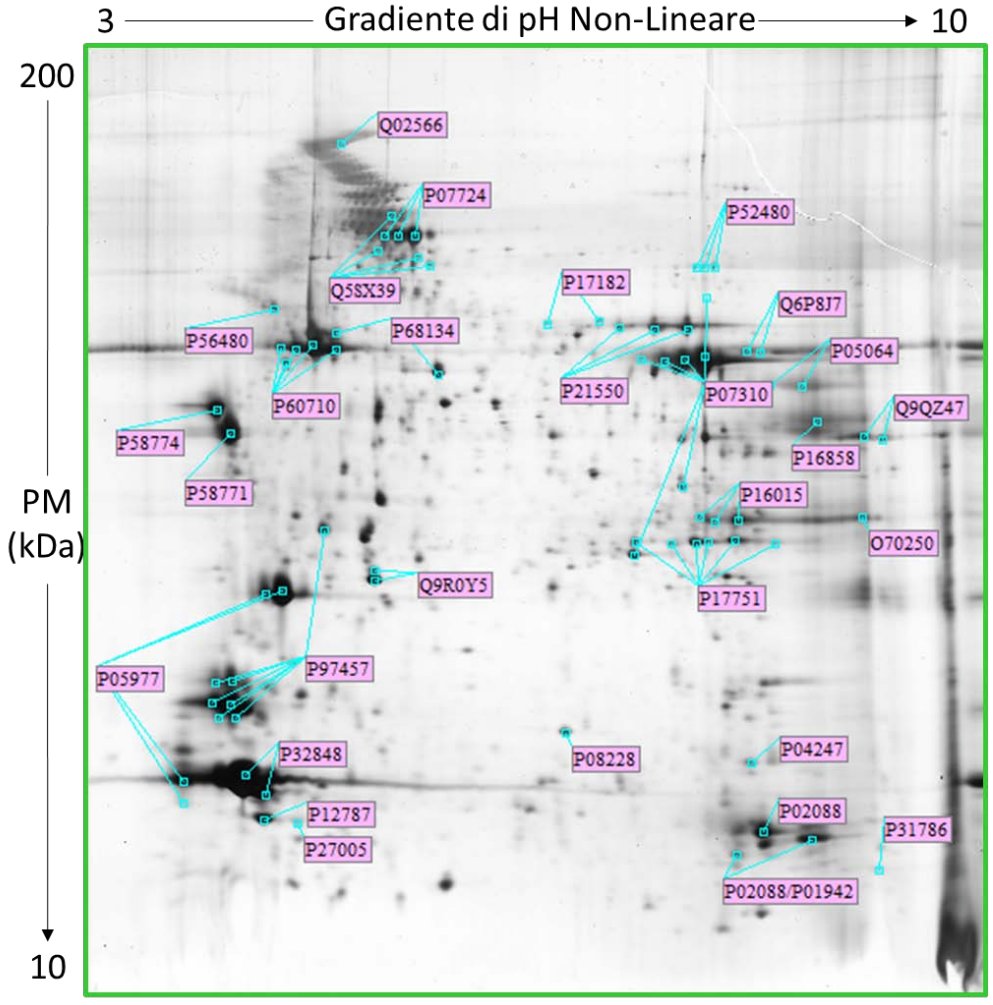


Fig.11.: *Proteomic map of quadriceps and protein spots identified*

It was also created a table in which each protein identified was associated:

- abbreviated name,
- number of access to the database,
- experimental pI and pM,
- mascot score, which is an index of statistical significance used in mass spectrometry,
- peptides identified,
- sequence scorage,
- identification technique, or gel matching with reference maps in specific databases, rather than peptide mass finger printing or mass-mass.

Nome Proteina	Nome abbreviato	Numero di accesso (Swiss-Prot) (a)	pI – MW (kDa) sperimentali (b)	Mascot score (c)	Peptidi identificati (d)	Sequence coverage % (e)	Tecnica di identificazione
Phosphoglycerate mutase 2	PGAM2	O70250	8,55 – 28208				GM(f)
Hemoglobin subunit beta-1	HBB1	P02088	7,20 – 11731	87	9	53	PMF(g)/MS-MS(h)
Hemoglobin subunit beta-1/Hemoglobin subunit alpha	HBB1/HBA	P02088/P01942	6,75 – 11852	48	6		PMF/MS-MS
Hemoglobin subunit beta-1/Hemoglobin subunit alpha		P02088/P01942	8,21 – 11770	25 - 26	3 – 4	25 – 50	GM - PMF/MS-MS
Myoglobin	MYG	P04247	7,13 – 13885				GM
Fructose-bisphosphate aldolase A	ALDOA	P05064	8,03 – 39728	209	16	59	GM – PMF
Fructose-bisphosphate aldolase A		P05064	7,33 – 40001	185	14	64	GM – PMF
Myosin light chain 1/3, skeletal muscle isoform	MYL1	P05977	4,50 – 12511				GM
Myosin light chain 1/3, skeletal muscle isoform		P05977	4,50 – 13044				GM
Myosin light chain 1/3, skeletal muscle isoform		P05977	4,96 – 23410				GM
Myosin light chain 1/3, skeletal muscle isoform		P05977	5,04 – 23161				GM
Creatine kinase M-type	KCRM	P07310	6,64 – 30091	92	9	28	PMF
Creatine kinase M-type		P07310	6,56 – 40985	142	12	44	GM – PMF
Creatine kinase M-type		P07310	6,46 – 40532				GM
Creatine kinase M-type		P07310	6,74 – 40352	158	13	43	GM - PMF/MS-MS
Creatine kinase M-type		P07310	6,65 – 40532	69	6	21	GM PMF/MS-MS
Creatine kinaseM-type		P07310	6,74 – 49922				MS-MS
Creatine kinase M-type		P07310	6,47 – 25176	73	9	27	MS-MS
Albumin	ALBU	P07724	5,35 – 66368				GM
Albumin		P07724	5,41 – 66000				GM
Albumin		P07724	5,35 – 66368				GM
Superoxide dismutase [Cu-Zn]	SODC	P08228	6,10 – 14597	82	5	31	PMF
Cytochrome c oxidase subunit 5A,	COX5A	P12787	5,12 –				GM

mitochondrial			10453				
Carbonic anhydrase 3		P16015	6,74 – 27613				GM
Carbonic anhydrase 3	CAH3	P16015	6,82 – 27782	78	7	48	PMF
Carbonic anhydrase 3		P16015	6,72 – 28122				GM - MS-MS
Glyceraldehyde-3-phosphate dehydrogenase	G3P	P16858	8,32 – 35785				GM
Alpha-enolase	ENOA	P17182	6,25 – 46511				GM
Alpha-enolase		P17182	6,01 – 46256				GM
Triosephosphate isomerase	TPIS	P17751	6,59 – 25605				GM
Triosephosphate isomerase		P17751	6,48 – 25667				GM
Triosephosphate isomerase		P17751	6,74 – 25667				GM
Triosephosphate isomerase		P17751	6,71 – 25636				GM
Triosephosphate isomerase		P17751	6,75 – 25824				GM
Triosephosphate isomerase		P17751	7,44 – 25636				GM

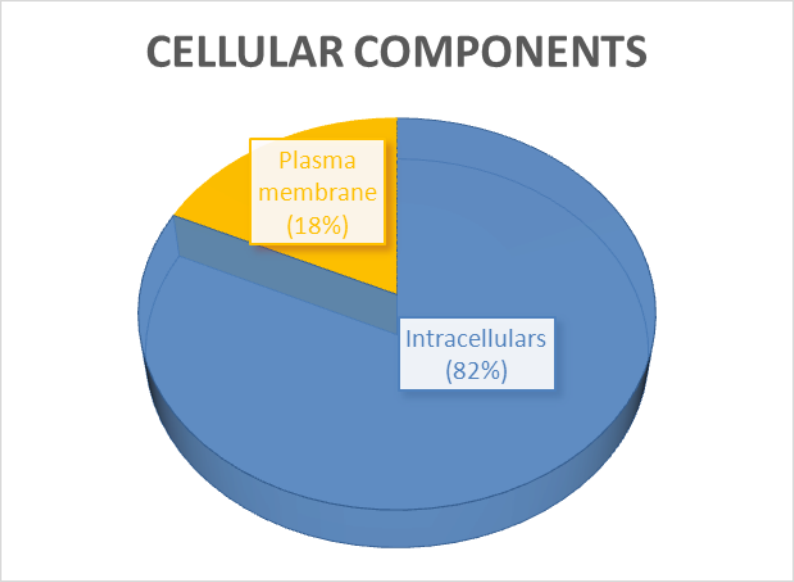
Beta-enolase	ENOB	P21550	6,52 - 4575(	68	9	20	GM – PMF
Beta-enolase		P21550	6,68 - 4575(				GM – PMF
Beta-enolase		P21550	6,35 - 4583				GM
Protein S100-A8	S100A8	P27005	5,14 - 1078(	56	4	73	PMF
Acyl-CoA-binding protein	ACBP	P31786	8,63 - 1249	35	3	45	PMF/MS-MS
Parvalbumin alpha	PRVA	P32848	4,96 - 1274	130	21	72	PMF
Parvalbumin alpha		P32848	4,84 - 1293	63	6	27	GM - PMF/MS-MS
Pyruvate kinase M	PKYM	P52480	6,74 - 5506				GM
Pyruvate kinase M		P52480	6,73 - 5568(				GM
Pyruvate kinase M		P52480	6,71 - 5506				GM
ATP synthase subunit beta, mitochondrial	ATBP	P56480	2,02 - 4780				GM
Tropomyosin alpha-1 chain	TPM1	P58771	4,87 - 3392				GM
Tropomyosin beta chain	TPM2	P58774	4,70 - 3632	89	16	33	PMF
Actin, cytoplasmic 1	ACTB	P60710	5,27 - 4293				GM
Actin, cytoplasmic 1		P60710	5,13 - 4317				GM
Actin, cytoplasmic 1		P60710	5,06 - 4341				GM
Actin, cytoplasmic 1		P60710	5,19 - 4372				GM
Actin, cytoplasmic 1		P60710	5,09 - 4135				GM
Actin, alpha skeletal muscle	ACTS	P68134	5,27 - 4520(				GM
Actin, alpha skeletal muscle		P68134	5,49 - 4125	69	6	19	PMF
Myosin regulatory light chain 2, skeletal muscle isoform	MLRS	P97457	5,23 - 2686	76	7	39	PMF
Myosin regulatory light chain 2, skeletal muscle isoform		P97457	4,77 - 1484				GM
Myosin regulatory light chain 2, skeletal muscle isoform		P97457	4,68 - 1496				GM
Myosin regulatory light chain 2, skeletal muscle isoform		P97457	4,78 – 1583	177		79	GM – PMF
Myosin regulatory light chain 2, skeletal muscle isoform		P97457	4,68 – 1590(	106		68	GM – PMF
Myosin regulatory light chain 2, skeletal muscle isoform		P97457	4,78 – 1748(	199	17	78	GM – PMF
Myosin regulatory light chain 2, skeletal muscle isoform		P97457	4,78 – 1748(	189	18	79	GM – PMF

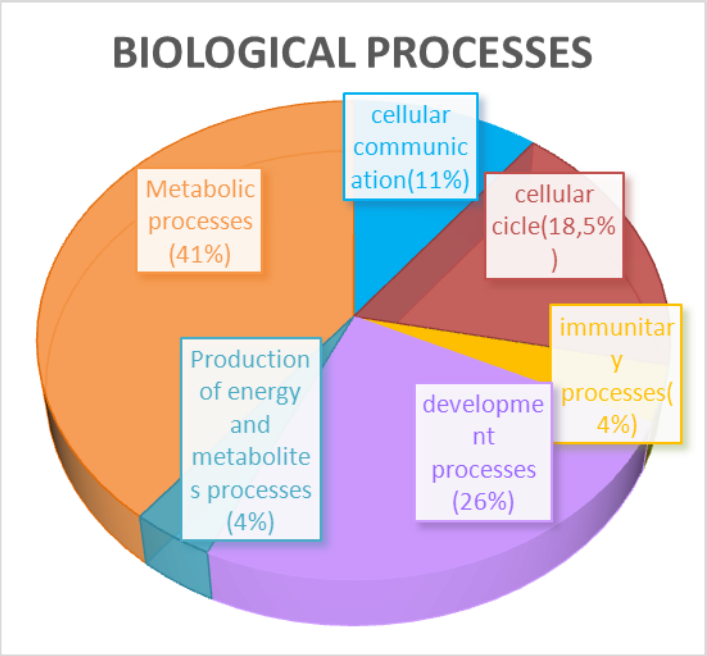
Myosin-6	MYH6	Q02566	5,27 –14192(				GM
Myosin-4		Q5SX39	5,40 – 4935'	119	25	11	PMF
Myosin-4		Q5SX39	5,44 – 5695(				PMF
Myosin-4	MYH4	Q5SX39	5,37 – 5915:				PMF
Myosin-4		Q5SX39	5,41 – 7431'	56	15	11	PMF/MS-MS
Creatine kinase S-type, mitochondrial	KCRS	Q6P8J7	7,18 – 4194:				GM
Creatine kinase S-type, mitochondrial		Q6P8J7	7,02 – 4227(				GM
Troponin T, fast skeletal muscle	TNNT3	Q9QZ47	8,65 – 3367:				PMF/MS-MS
Troponin T, fast skeletal muscle		Q9QZ47	8,56 – 3367:	75	8	26	PMF/MS-MS
Adenylate kinase isoenzyme 1	KAD1	Q9R0Y5	5,33 – 2429'	88	9	51	PMF
Adenylate kinase isoenzyme 1		Q9R0Y5	5,33 – 2372(	100	10	48	PMF

Table 3. : *Table that shows the 31 proteins identified in the reference proteomic quadriceps map.*

In order to have a functional enhancement of the identified proteins, we wanted to make a subdivision of the same, according to the three criteria of the *Gene Ontology*: cellular components, molecular functions, biological processes.

Thanks to this analysis was possible to ascertain that 82% are cellular proteins, most of which has both cellular functions that enzymatic functions and another part of them is involved in metabolic processes.





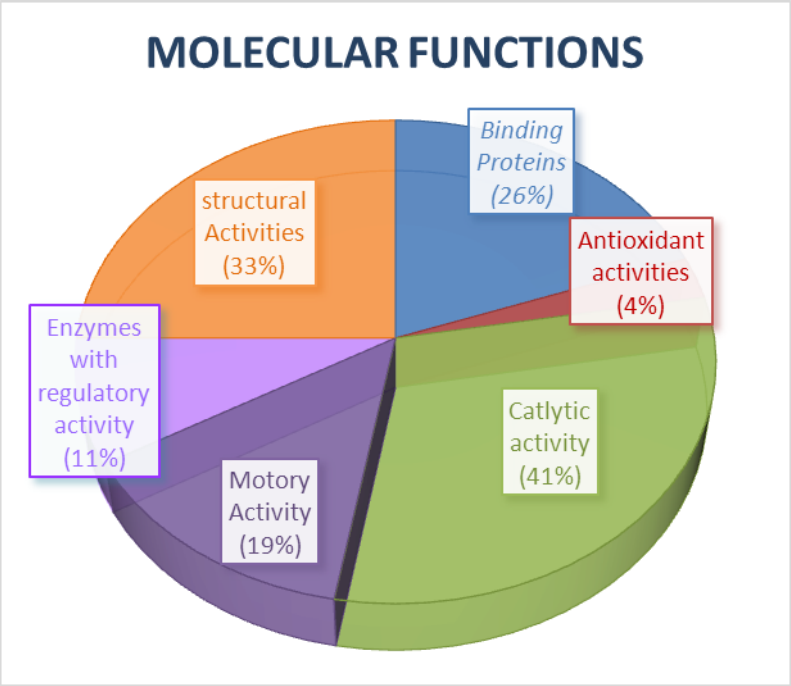


Fig.12,13 and 14 : *Graphs obtained from the analysis carried out by Gene Onthology*

This type of analysis was further confirmed by analysis of the classes of the system *panther protein*, which indicates that most of the protein has a role purely enzymatic and structural.

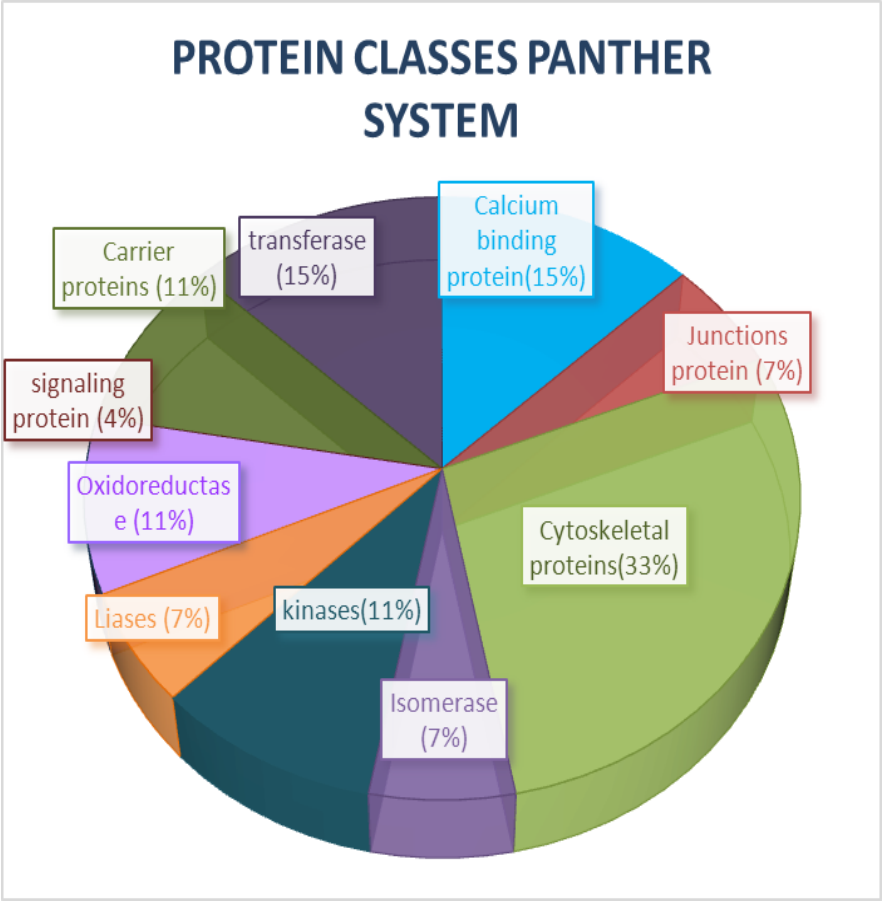
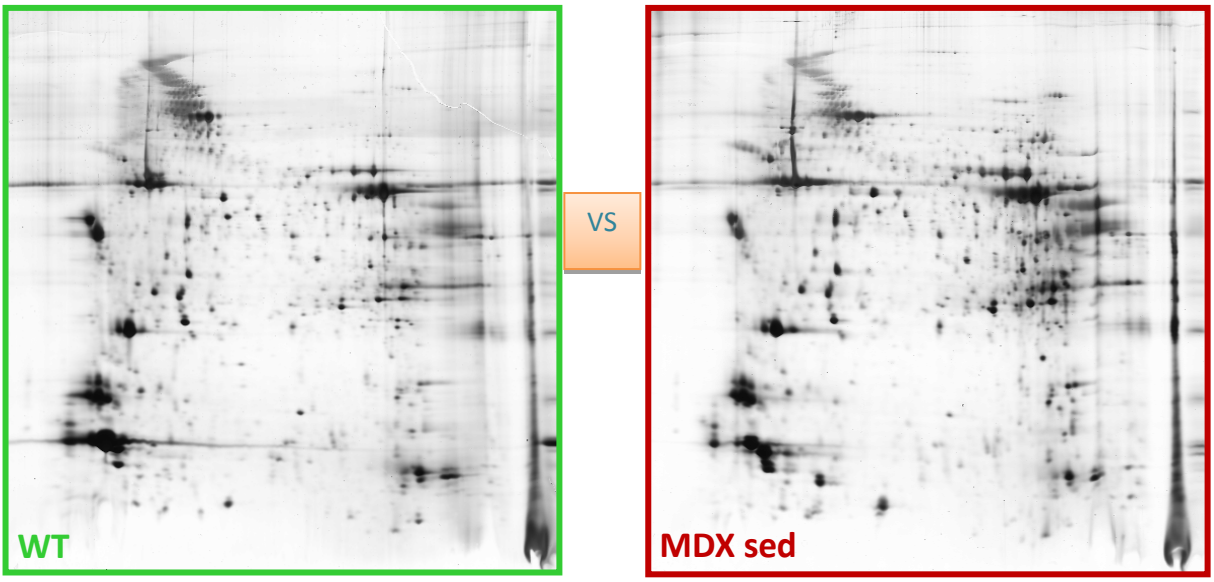


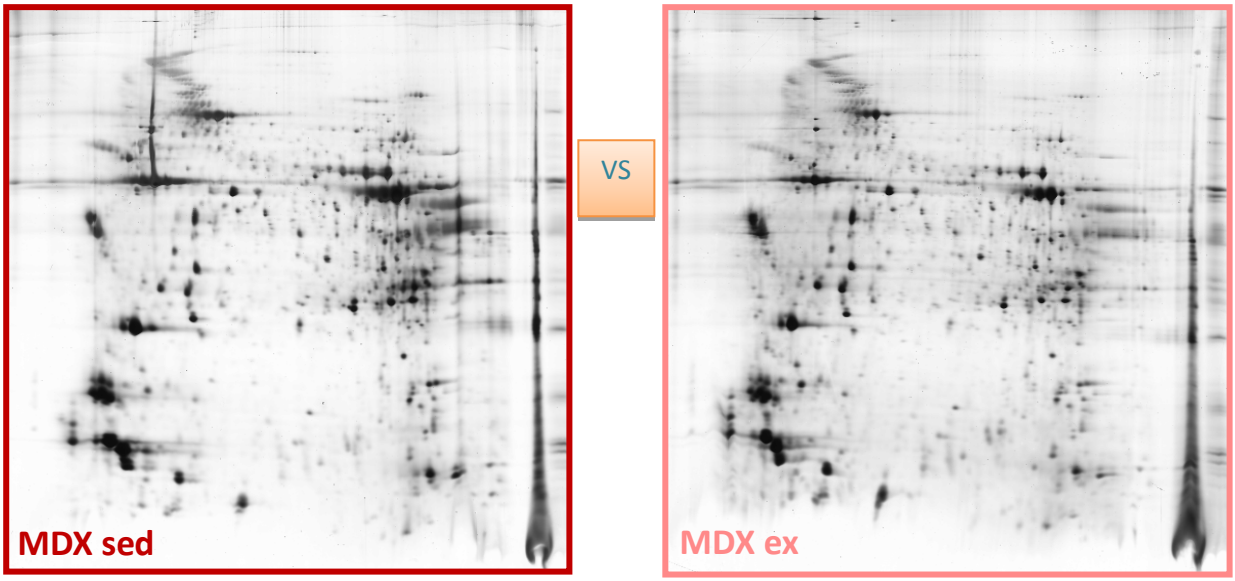
Fig.15: *Protein classes panther system*

Comparative Analysis

It was conducted comparative analysis of 2D gel scanned using analytical software bioinformatics, in this case Image Master 2D platinum. This software allows you to perform quantitative analyzes related to different proteomic profiles. Were made two types of comparison:

- proteomic profile of wild type mice versus mdx not trained mice;
- proteomic profile of mdx trained mice versus mdx not trained mice.





The software, in order to make on quantitative analysis, using as a parameter the volume percentage. This volume is obtained from the volume of a given spot, divided by the summation of all the volumes in a specific map for 100. This calculation was performed in order to have a more normalized data possible and make accurate analysis.

Experimental replicates

In order to perform accurate analysis of proteomics is strictly necessary to follow a precise experimental design: it was performed a minimum of three experimental replicates each condition examined, from which it is evidenced that there was no significant difference.

Therefore, the study was based on a minimum of three independent replicates for each condition tested: wild type mice, mdx mice trained and not trained mdx mice. The parameter used for the analysis of the maps was the volume percentage, obtained from an average of at least three independent experimental replicates for each sample.

P-cluster analysis

To have a global view of changes in the pattern of protein expression of the three conditions examined, hierarchical cluster analysis was performed by the use of specific algorithms such as p-cluster analysis. From this analysis it was possible to observe that the two proteomic profiles of mdx mice are more similar to each other than the proteomic profile of the wild-type mice, and this has suggested that exercise does not result in a drastic change in the proteomic profile of the mdx.

This global analysis is confirmed by a detailed analysis of comparative proteomic.

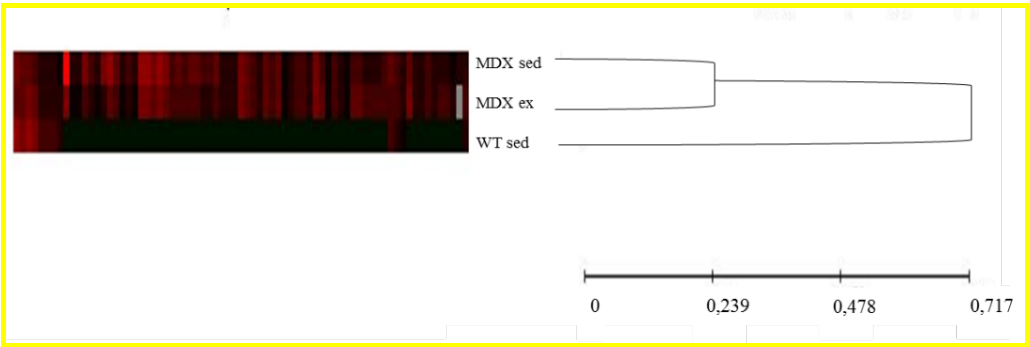


Fig.16: *P-cluster analysis of the proteins*

Proteomic comparison profile wild-type mice versus mdx mice not trained

In the comparison of the analytical maps of the quadriceps of wt mice versus mdx not trained, we observed 12 spots corresponding to differentially expressed proteins involved in carbohydrate metabolism as the triosofosfatoisomerasi, glyceraldehyde-3-phosphate dehydrogenase, and proteins involved in muscle contraction such as the myosin light chain, proteins that play a role in the mechanism regulation of oxidative stress.

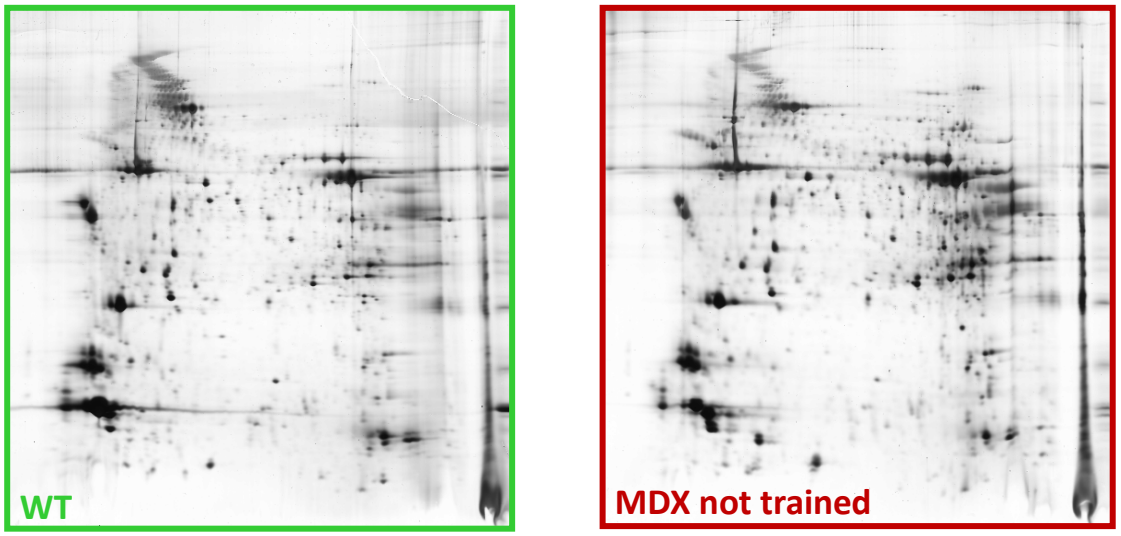


Fig.17 and 18: *Proteomic maps of the two proteomes examined: wild tipe mice and dystrophic mice.*

In the table below we show the 12 differentially expressed proteins in the two proteomes examined.

Nome Proteina	Nome abbreviato	N. di accesso in Swiss-Prot®	pI-PM (kDa)	MDX sed *vs* WT sed (a)	t-test (a)	MDX ex *vs* MDX sed (a)	t-test (a)
Carbonic anhydrase 3	CAH3	P16015	6,72 - 28122	2,8	0,046	-3,6	0,022
Carbonic anhydrase 3	CAH3	P16015	6,74 - 27613	2,7	0,040	-2,3	0,042
Carbonic anhydrase 3	CAH3	P16015	6,82 - 27782	2,1	0,033	-1,7	0,039
Cytochrome c oxidase subunit 5A, mitochondrial	COX5A	P12787	5,12 - 10453	-2,5	0,047		
Glyceraldehyde-3-phosphate dehydrogenase	G3P	P16858	8,32 - 35785	1,7	0,034		
Myosin regulatory light chain 2, skeletal muscle isoform	MLRS	P97457	4,78 - 15834	-7,9	0,005		
Myosin 4	MYH4	Q5SX39	5,37 - 59155	-4,3	0,027		

Myosin light chain 1/3, skeletal muscle isoform	MYL1	P05977	4,50 - 13044	-4,1	0,043		
Parvalbumin alpha	PRVA	P32848	4,84 - 12935	-4,7	0,013		
Superoxide dismutase [Cu-Zn]	SODC	P08228	6,10 - 14597	-5,28	0,02	2,10	0,01
Troponin T	TNNT	Q9QZ47	8,65 - 33679	-3,1	0,020		
Triosephosphate isomerase	TPIS	P17751	6,48 25667	1,5	0,014		

Table4: *The table shows the 12 different proteins expressed in the two proteomes examined, wt mice and mdx not trained mice.*

Proteomic comparison profile mdx trained mice versus mdx not trained mice .

In the comparison between the maps of mdx mice trained and not trained mdx mice, we observed only 4 spots differentially expressed, which correspond to the three isoforms of carbonic anhydrase and superoxide dismutase-c. This is of particular biological significance because the semi-quantitative differences observed between mdx mdx mice trained and not trained and wild type mice, are changed back by aerobic exercise of short duration and low intensity. Was considered a significant change when it is obtained a variation of at least 1, 5 times and a p-value ≤0.05.

Nome Proteina	Nome abbreviato	N. di accesso in Swiss-Prot®	pI-PM (kDa)	MDX sed *vs* WT sed (a)	t-test (a)	MDX ex *vs* MDX sed (a)	t-test (a)
Carbonic anhydrase 3	CAH3	P16015	6,72 - 28122	2,8	0,046	-3,6	0,022
Carbonic anhydrase 3	CAH3	P16015	6,74 - 27613	2,7	0,040	-2,3	0,042
Carbonic anhydrase 3	CAH3	P16015	6,82 - 27782	2,1	0,033	-1,7	0,039
Superoxide dismutase [Cu-Zn]	SODC	P08228	6,10 - 14597	-5,28	0,02	2,10	0,01

Table 5: *Table shows the 4 proteins differentially expressed in the two proteomes examined*

Changes of superoxide dismutase and carbonic anhydrase-3

Going to see the specific changes of superoxide dismutase-c, it is possible to note from the graph below, which are witnessing a substantial decrease in the levels of this protein compared to wt and in phenotype mdx trained mice.

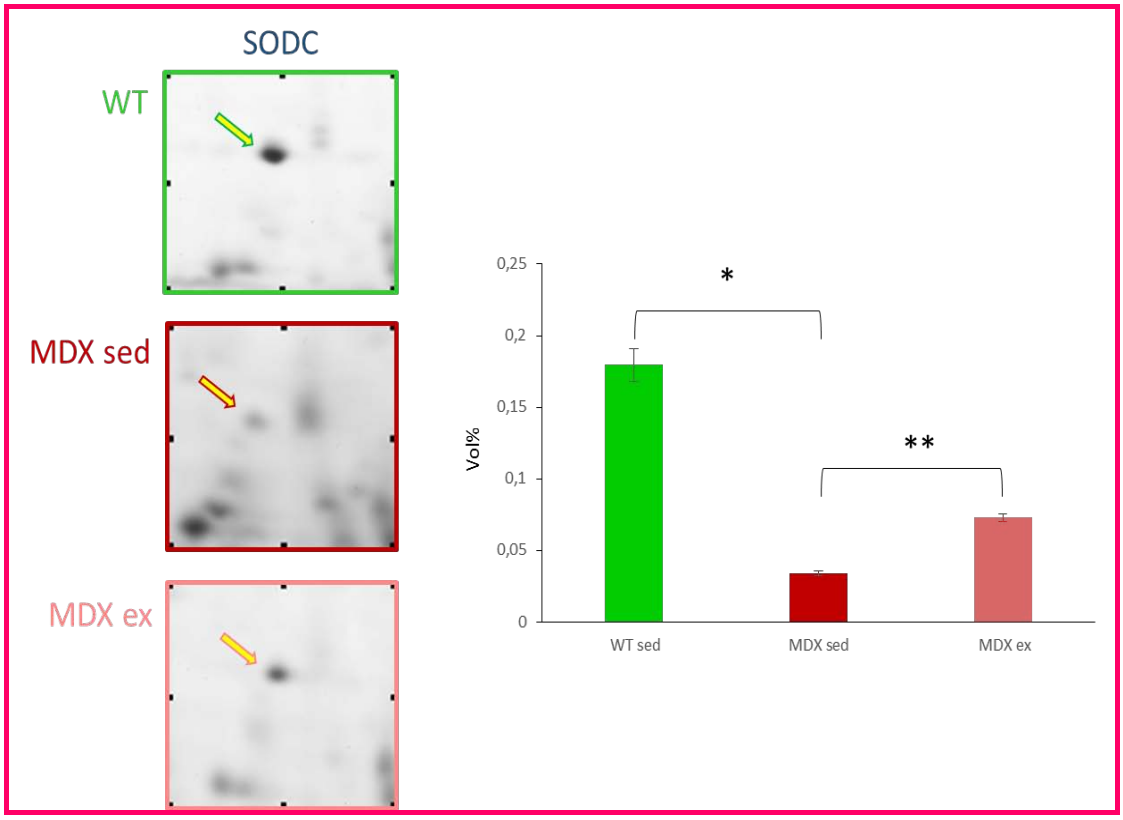


Fig.19 : *The graph shows the different expression of the SODC protein in the three proteomes examined. The protein is highly expressed in the phenotype wt, decreases in the phenotype of mdx mice not trained, but it is very expressed in the mdx phenotype trained.*

The sod-c is an enzyme with antioxidant activity involved in the regulation of oxidative stress and having found a decrease of that protein in dystrophic fibers not subjected to training, we are witnessing an increase in oxidative stress in these fibers, which is a typical characteristic of dystrophic muscle fibers.

This increase in oxidative stress, may also explain the increase of the three isoforms of carbonic anhydrase that is we have observed in the phenotype of mdx mice not trained.

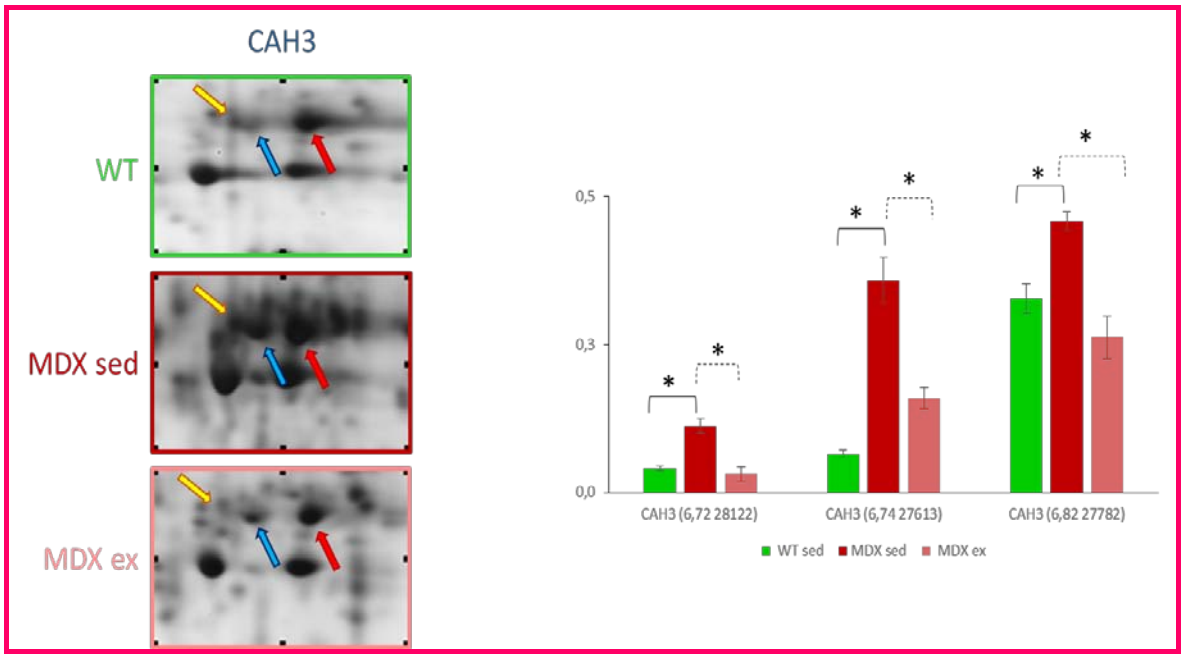


Fig 20: *The graph shows the different expression of the CAH3 protein in the three proteomes examined. The protein is very expressed in the phenotype mdx not trained.*

The carbonic anhydrase 3 are metallo-enzymes involved in biological processes and has been shown to have a role in the regulation of oxidative stress. In the literature, in some work, has been shown that there is a correlation directly proportional between increase in oxidative stress in the dystrophic fibers and increased levels of CAH3.

In the context of innovative therapies such as antioxidant therapies, has been shown that by using specific inhibitors such as those for CAH3 precisely in mdx mice, we are witnessing a decrease of the areas of necrosis and a functional recovery of the muscle itself.

Discussion

Recovery of damaged skeletal muscle following low-intensity endurance exercise and proteomics analysis

Based on an previous study conducted in our laboratory, (Frinchi et al 2013) in which it is shown that an appropriate training program of short duration and low intensity leads to an acceleration in the process of muscle regeneration in dystrophic mice, we wanted to investigate the possibility that the 'training, could perform the same role in an experimental model that is not present muscle damage related to a disease, but rather, to a muscle trauma of mechanical origin.

To conduct our study, were used rats to which was produced traumatic muscle damage and were subsequently subjected to a period of endurance training. To evaluate the process of muscle regeneration from training accelerated endurance were evaluated expression levels of CX39 (Belluardo 2005) as a marker of tissue regeneration. The study conducted shows that the levels of this protein, expressed in the early stages of embryonic life and after muscle damage, tend to be lower in rats with crush undergo training compared with rats with crush not undergo training.

The fact that the levels of CX39 are lower in rats with crush undergoing training, indicates that in muscle take place healing process favored by training. Concerning the mechanisms involved in this recovery of muscle damage, we may examine several possibilities.

Endurance training produces many physiological, metabolic and vascular adaptations in skeletal muscle but it is not clear how these changes may improve muscle damage . Low-intensity endurance may have anti-inflammatory action that can prevent or reduce muscle degeneration process in rats as has seen in a previous study in mdx mice. More likely, several factors released in response to endurance exercise may regulate muscle stem cells and therefore enhance the adaptive response of skeletal muscle.

Based on the results obtained from experiments on rats, we wondered what were the molecular mechanisms that underlie this process of accelerated muscle regeneration from training endurance. So we conducted a proteomics analysis in order to have a window open on muscle cells at a particular time in their lives.

To make the proteomic analysis, we used the mdx mice as an experimental model, previously studied in our laboratory.(Frinchi et al).

The mdx mice were divided into three experimental groups, wt Mdx, Mdx trained and not trained Mdx. Of these animals, we have built the proteome reference map of the quadriceps muscle. This map was not yet present in the literature.

The map identified 73 protein spots corresponding to 31 proteins. Once obtained the reference map was possible to conduct comparative proteomic analysis, comparing the three different proteomes. From this analysis it was found that in the three proteomes varies very expression of proteins involved in the mechanism of oxidative stress.

From this analysis it was found that in the three proteomes varies very expression of proteins involved in the mechanism of oxidative stress. In particular vary four proteins, namely, the three isoforms of carbonic anhydrase and superoxide dismutase-c.

Looking specifically Sod-c, from the analysis we have seen a decrease in the levels of this protein in the mdx phenotype not trained. Instead, this protein is highly expressed in the phenotype of mdx be trained. As we have seen, the Sod-c is a metal- enzyme with antioxidant activity involved in the regulation of oxidative stress. A decrease in the levels of expression of this protein in the fibers dystrophic untrained, involves an increase in oxidative stress in these fibers.

Such an increase in oxidative stress may also explain the increased expression of the three isoforms of carbonic anhydrase, which, as shown by the analysis conducted, it is observed in the mdx phenotype not trained. The analysis shows that there are obvious differences compared to the wt phenotype, the phenotype mdx trained is closer to the phenotype wt.

In some studies in the literature, it has been demonstrated that there is a directly proportional correlation between increased oxidative stress in these fibers and increase of carbonic anhydrase-3. In the literature in the context of innovative therapies, therapies such as antioxidants, it has been shown that by using specific inhibitors such as those for CAH3 in mdx mice is witnessing a decrease of the areas of necrosis. So, not only pharmacologically but also as we have shown exercise, contributes to functional recovery of the muscle.

Reference List

Amthor H, Huang R, McKinnell I, Christ B, Kambadur R, Sharma M, Patel K (The regulation and action of myostatin as a negative regulator of muscle development during avian embryogenesis. Dev Biol 251:241-257.2002).

Armulik A, Abramsson A, Betsholtz C (Endothelial/pericyte interactions. Circ Res 97:512-523.2005).

Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA (Myogenic specification of side population cells in skeletal muscle. J Cell Biol 159:123-134.2002).

Auerbach AA, Bennett MV (Chemically mediated transmission at a giant fiber synapse in the central nervous system of a vertebrate. J Gen Physiol 53:183-210.1969).

Balogh S, Naus CC, Merrifield PA (Expression of gap junctions in cultured rat L6 cells during myogenesis. Dev Biol 155:351-360.1993).

Belluardo N, Mudo G, Trovato-Salinaro A, Le GS, Charollais A, Serre-Beinier V, Amato G, Haefliger JA, Meda P, Condorelli DF (Expression of connexin36 in the adult and developing rat brain. Brain Res 865:121-138.2000).

Belluardo N, Trovato-Salinaro A, Mudo G, Condorelli DF (Expression of the rat connexin 39 (rCx39) gene in myoblasts and myotubes in developing and regenerating skeletal muscles: an in situ hybridization study. Cell Tissue Res 320:299-310.2005).

Bennett MV, Verselis VK (Biophysics of gap junctions. Semin Cell Biol 3:29-47.1992).

Blaveri K, Heslop L, Yu DS, Rosenblatt JD, Gross JG, Partridge TA, Morgan JE (Patterns of repair of dystrophic mouse muscle: studies on isolated fibers. Dev Dyn 216:244-256.1999).

Boldrin L, Morgan JE (Activating muscle stem cells: therapeutic potential in muscle diseases. Curr Opin Neurol 20:577-582.2007).

Brunelli S, Sciorati C, D'Antona G, Innocenzi A, Covarello D, Galvez BG, Perrotta C, Monopoli A, Sanvito F, Bottinelli R, Ongini E, Cossu G, Clementi E (Nitric oxide release combined with nonsteroidal antiinflammatory activity prevents muscular dystrophy pathology and enhances stem cell therapy. Proc Natl Acad Sci U S A 104:264-269.2007).

Brussee V, Tardif F, Tremblay JP (Muscle fibers of mdx mice are more vulnerable to exercise than those of normal mice. Neuromuscul Disord 7:487-492.1997).

Bruzzone R, Meda P (The gap junction: a channel for multiple functions? Eur J Clin Invest 18:444-453.1988).

Bruzzone R, White TW, Goodenough DA (The cellular Internet: on-line with connexins. Bioessays 18:709-718.1996a).

Bruzzone R, White TW, Paul DL (Connections with connexins: the molecular basis of direct intercellular signaling. Eur J Biochem 238:1-27.1996b).

Bulfield G, Siller WG, Wight PA, Moore KJ (X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A 81:1189-1192.1984).

Burdi R, Didonna MP, Pignol B, Nico B, Mangieri D, Rolland JF, Camerino C, Zallone A, Ferro P, Andreetta F, Confalonieri P, De LA (First evaluation of the potential effectiveness in muscular dystrophy of a novel chimeric compound, BN 82270, acting as calpain-inhibitor and anti-oxidant. Neuromuscul Disord 16:237-248.2006).

Burkin DJ, Wallace GQ, Milner DJ, Chaney EJ, Mulligan JA, Kaufman SJ (Transgenic expression of {alpha}7{beta}1 integrin maintains muscle integrity, increases regenerative capacity, promotes hypertrophy, and reduces cardiomyopathy in dystrophic mice. Am J Pathol 166:253-263.2005).

Carter GT, Wineinger MA, Walsh SA, Horasek SJ, Abresch RT, Fowler WM, Jr. (Effect of voluntary wheel-running exercise on muscles of the mdx mouse. Neuromuscul Disord 5:323-332.1995).

Caveney S (The role of gap junctions in development. Annu Rev Physiol 47:319-335.1985).

Charge SB, Rudnicki MA (Cellular and molecular regulation of muscle regeneration. Physiol Rev 84:209-238.2004).

Chazaud B, Sonnet C, Lafuste P, Bassez G, Rimaniol AC, Poron F, Authier FJ, Dreyfus PA, Gherardi RK (Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. J Cell Biol 163:1133-1143.2003).

Christov C, Chretien F, bou-Khalil R, Bassez G, Vallet G, Authier FJ, Bassaglia Y, Shinin V, Tajbakhsh S, Chazaud B, Gherardi RK (Muscle satellite cells and endothelial cells: close neighbors and privileged partners. Mol Biol Cell 18:1397-1409.2007).

Condorelli DF, Belluardo N, Trovato-Salinaro A, Mudo G (Expression of Cx36 in mammalian neurons. Brain Res Brain Res Rev 32:72-85.2000).

Condorelli DF, Trovato-Salinaro A, Mudo G, Mirone MB, Belluardo N (Cellular expression of connexins in the rat brain: neuronal localization, effects of kainate-induced seizures and expression in apoptotic neuronal cells. Eur J Neurosci 18:1807-1827.2003).

Constantin B, Cronier L (Involvement of gap junctional communication in myogenesis. Int Rev Cytol 196:1-65.2000).

Corbel SY, Lee A, Yi L, Duenas J, Brazelton TR, Blau HM, Rossi FM (Contribution of hematopoietic stem cells to skeletal muscle. Nat Med 9:1528-1532.2003).

Cornelison DD, Wold BJ (Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191:270-283.1997).

Cossu G, Bianco P (Mesoangioblasts--vascular progenitors for extravascular mesodermal tissues. Curr Opin Genet Dev 13:537-542.2003).

De Mello WC (Gap junctional communication in excitable tissues; the heart as a paradigma. Prog Biophys Mol Biol 61:1-35.1994).

Decary S, Mouly V, Hamida CB, Sautet A, Barbet JP, Butler-Browne GS (Replicative potential and telomere length in human skeletal muscle: implications for satellite cell-mediated gene therapy. Hum Gene Ther 8:1429-1438.1997).

Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, Innocenzi A, Galvez BG, Messina G, Morosetti R, Li S, Belicchi M, Peretti G, Chamberlain JS, Wright WE, Torrente Y, Ferrari S, Bianco P, Cossu G (Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol 9:255-267.2007).

Doran P, Martin G, Dowling P, Jockusch H, Ohlendieck K (Proteome analysis of the dystrophin-deficient MDX diaphragm reveals a drastic increase in the heat shock protein cvHSP. Proteomics 6:4610-4621.2006).

Durbeej M, Campbell KP (Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. Curr Opin Genet Dev 12:349-361.2002).

El FE, Torrente Y, Caron NJ, Bresolin MD, Tremblay JP (In vivo migration of transplanted myoblasts requires matrix metalloproteinase activity. Exp Cell Res 258:279-287.2000).

Emery AE (The muscular dystrophies. Lancet 359:687-695.2002).

Farini A, Razini P, Erratico S, Torrente Y, Meregalli M (Cell based therapy for Duchenne muscular dystrophy. J Cell Physiol 221:526-534.2009).

Ferrari G, Cusella-De AG, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528-1530.1998).

FURSHPAN EJ, POTTER DD (Transmission at the giant motor synapses of the crayfish. J Physiol 145:289-325.1959).

Gargioli C, Coletta M, De GF, Cannata SM, Cossu G (PlGF-MMP-9-expressing cells restore microcirculation and efficacy of cell therapy in aged dystrophic muscle. Nat Med 14:973-978.2008).

Gopinath SD, Rando TA (Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell 7:590-598.2008).

Gordon KJ, Blobe GC (Role of transforming growth factor-beta superfamily signaling pathways in human disease. Biochim Biophys Acta 1782:197-228.2008).

Grefte S, Kuijpers-Jagtman AM, Torensma R, Von den Hoff JW (Skeletal muscle development and regeneration. Stem Cells Dev 16:857-868.2007).

Grounds MD, Radley HG, Lynch GS, Nagaraju K, De LA (Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. Neurobiol Dis 31:1-19.2008).

Grounds MD, Torrisi J (Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis. FASEB J 18:676-682.2004).

Guthrie SC, Gilula NB (Gap junctional communication and development. Trends Neurosci 12:12-16.1989).

Hawke TJ, Garry DJ (Myogenic satellite cells: physiology to molecular biology. J Appl Physiol (1985) 91:534-551.2001).

Hayes A, Lynch GS, Williams DA (The effects of endurance exercise on dystrophic mdx mice. I. Contractile and histochemical properties of intact muscles. Proc Biol Sci 253:19-25.1993).

Hayes A, Williams DA (Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle. J Appl Physiol (1985) 80:670-679.1996).

Hayes A, Williams DA (Contractile function and low-intensity exercise effects of old dystrophic (mdx) mice. Am J Physiol 274:C1138-C1144.1998).

Hodgetts S, Radley H, Davies M, Grounds MD (Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. Neuromuscul Disord 16:591-602.2006).

Hoffman EP, Dressman D (Molecular pathophysiology and targeted therapeutics for muscular dystrophy. Trends Pharmacol Sci 22:465-470.2001).

Huard J, Li Y, Fu FH (Muscle injuries and repair: current trends in research. J Bone Joint Surg Am 84-A:822-832.2002).

Irintchev A, Zeschnigk M, Starzinski-Powitz A, Wernig A (Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev Dyn 199:326-337.1994).

Jaumot M, Estanol JM, Casanovas O, Grana X, Agell N, Bachs O (The cell cycle inhibitor p21CIP is phosphorylated by cyclin A-CDK2 complexes. Biochem Biophys Res Commun 241:434-438.1997).

Kherif S, Lafuma C, Dehaupas M, Lachkar S, Fournier JG, Verdiere-Sahuque M, Fardeau M, Alameddine HS (Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: a study in experimentally injured and mdx muscles. Dev Biol 205:158-170.1999).

Klingler W, Jurkat-Rott K, Lehmann-Horn F, Schleip R (The role of fibrosis in Duchenne muscular dystrophy. Acta Myol 31:184-195.2012).

Kuang S, Gillespie MA, Rudnicki MA (Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2:22-31.2008).

Lovering RM, Porter NC, Bloch RJ (The muscular dystrophies: from genes to therapies. Phys Ther 85:1372-1388.2005).

Malerba A, Vitiello L, Segat D, Dazzo E, Frigo M, Scambi I, De CP, Boldrin L, Martelli L, Pasut A, Romualdi C, Bellomo RG, Vecchiet J, Baroni MD (Selection of multipotent cells and enhanced muscle reconstruction by myogenic macrophage-secreted factors. Exp Cell Res 315:915-927.2009).

Manzur AY, Kuntzer T, Pike M, Swan A (Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev:CD003725.2008).

MAURO A (Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493-495.1961).

Maxeiner S, Kruger O, Schilling K, Traub O, Urschel S, Willecke K (Spatiotemporal transcription of connexin45 during brain development results in neuronal expression in adult mice. Neuroscience 119:689-700.2003).

McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R (Myostatin negatively regulates satellite cell activation and self-renewal. J Cell Biol 162:1135-1147.2003).

Miller SM, Garfield RE, Daniel EE (Improved propagation in myometrium associated with gap junctions during parturition. Am J Physiol 256:C130-C141.1989).

Minasi MG, Riminucci M, De AL, Borello U, Berarducci B, Innocenzi A, Caprioli A, Sirabella D, Baiocchi M, De MR, Boratto R, Jaffredo T, Broccoli V, Bianco P, Cossu G (The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development 129:2773-2783.2002).

Muntoni F (Cardiomyopathy in muscular dystrophies. Curr Opin Neurol 16:577-583.2003).

Odom GL, Gregorevic P, Chamberlain JS (Viral-mediated gene therapy for the muscular dystrophies: successes, limitations and recent advances. Biochim Biophys Acta 1772:243-262.2007).

Ohlstein B, Kai T, Decotto E, Spradling A (The stem cell niche: theme and variations. Curr Opin Cell Biol 16:693-699.2004).

Okano T, Yoshida K, Nakamura A, Sasazawa F, Oide T, Takeda S, Ikeda S (Chronic exercise accelerates the degeneration-regeneration cycle and downregulates insulin-like growth factor-1 in muscle of mdx mice. Muscle Nerve 32:191-199.2005).

Page E, Shibata Y (Permeable junctions between cardiac cells. Annu Rev Physiol 43:431-441.1981).

Philippou A, Maridaki M, Halapas A, Koutsilieris M (The role of the insulin-like growth factor 1 (IGF-1) in skeletal muscle physiology. In Vivo 21:45-54.2007).

Proulx A, Merrifield PA, Naus CC (Blocking gap junctional intercellular communication in myoblasts inhibits myogenin and MRF4 expression. Dev Genet 20:133-144.1997).

Radley HG, De LA, Lynch GS, Grounds MD (Duchenne muscular dystrophy: focus on pharmaceutical and nutritional interventions. Int J Biochem Cell Biol 39:469-477.2007).

Rando TA, Disatnik MH, Yu Y, Franco A (Muscle cells from mdx mice have an increased susceptibility to oxidative stress. Neuromuscul Disord 8:14-21.1998).

Schmalbruch H (Skeletal muscle fibers of newborn rats are coupled by gap junctions. Dev Biol 91:485-490.1982).

Schultz E, McCormick KM (Skeletal muscle satellite cells. Rev Physiol Biochem Pharmacol 123:213-257.1994).

Seale P, Rudnicki MA (A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev Biol 218:115-124.2000).

Segawa M, Fukada S, Yamamoto Y, Yahagi H, Kanematsu M, Sato M, Ito T, Uezumi A, Hayashi S, Miyagoe-Suzuki Y, Takeda S, Tsujikawa K, Yamamoto H (Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. Exp Cell Res 314:3232-3244.2008).

Serrano AL, Munoz-Canoves P (Regulation and dysregulation of fibrosis in skeletal muscle. Exp Cell Res 316:3050-3058.2010).

Serre-Beinier V, Le GS, Belluardo N, Trovato-Salinaro A, Charollais A, Haefliger JA, Condorelli DF, Meda P (Cx36 preferentially connects beta-cells within pancreatic islets. Diabetes 49:727-734.2000).

Shi X, Garry DJ (Muscle stem cells in development, regeneration, and disease. Genes Dev 20:1692-1708.2006).

Soehnlein O, Lindbom L (Phagocyte partnership during the onset and resolution of inflammation. Nat Rev Immunol 10:427-439.2010).

Sotelo C, Llinas R (Specialized membrane junctions between neurons in the vertebrate cerebellar cortex. J Cell Biol 53:271-289.1972).

Spencer MJ, Mellgren RL (Overexpression of a calpastatin transgene in mdx muscle reduces dystrophic pathology. Hum Mol Genet 11:2645-2655.2002).

Spray DC, Burt JM (Structure-activity relations of the cardiac gap junction channel. Am J Physiol 258:C195-C205.1990).

Squecco R, Sassoli C, Nuti F, Martinesi M, Chellini F, Nosi D, Zecchi-Orlandini S, Francini F, Formigli L, Meacci E (Sphingosine 1-phosphate induces myoblast differentiation through Cx43 protein expression: a role for a gap junction-dependent and -independent function. Mol Biol Cell 17:4896-4910.2006).

Tidball JG, Wehling-Henricks M (Evolving therapeutic strategies for Duchenne muscular dystrophy: targeting downstream events. Pediatr Res 56:831-841.2004).

Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G, Tonlorenzi R, Porretti L, Gavina M, Mamchaoui K, Pellegrino MA, Furling D, Mouly V, Butler-Browne GS, Bottinelli R, Cossu G, Bresolin N (Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest 114:182-195.2004).

Torrente Y, El FE, Caron NJ, Bresolin N, Tremblay JP (Intramuscular migration of myoblasts transplanted after muscle pretreatment with metalloproteinases. Cell Transplant 9:539-549.2000).

Trovato-Salinaro A, Belluardo N, Frinchi M, von MJ, Willecke K, Condorelli DF, Mudo G (Regulation of connexin gene expression during skeletal muscle regeneration in the adult rat. Am J Physiol Cell Physiol 296:C593-C606.2009).

Tyler KL (Origins and early descriptions of "Duchenne muscular dystrophy". Muscle Nerve 28:402-422.2003).

Uezumi A, Ojima K, Fukada S, Ikemoto M, Masuda S, Miyagoe-Suzuki Y, Takeda S (Functional heterogeneity of side population cells in skeletal muscle. Biochem Biophys Res Commun 341:864-873.2006).

Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG (Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Hum Mol Genet 18:482-496.2009).

von MJ, Euwens C, Willecke K, Sohl G (The novel mouse connexin39 gene is expressed in developing striated muscle fibers. J Cell Sci 117:5381-5392.2004).

Yablonka-Reuveni Z, Rivera AJ (Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev Biol 164:588-603.1994).

Zammit PS, Partridge TA, Yablonka-Reuveni Z (The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem 54:1177-1191.2006a).

Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, Beauchamp JR (Pax7 and myogenic progression in skeletal muscle satellite cells. J Cell Sci 119:1824-1832.2006b).

Zhao P, Hoffman EP (Embryonic myogenesis pathways in muscle regeneration. Dev Dyn 229:380-392.2004).